REMARKS

The claims are 1 to 11.

Undersigned acknowledges with appreciation the indication that claims 2 to 10 would be allowable if rewritten in independent form. However, for reasons set forth below, it is considered that all of the claims in this application are now in condition for allowance.

With regard to the rejection of claim 1 under 35 U.S.C. 112, the point of attachment for substituent R^2 is now specified in accordance with the disclosure on page 8, lines 13 to 27, among other locations in the specification.

With regard to the rejection of claim 11 on the ground of lack of enablement, claim 11 now specifies specific diseases in which other compounds which have p38MAP kinase-inhibiting activity effective against, e.g., rheumatoid arthritis and IBD.

In support of this fact, please see 1) J. Pharmacol. Exp. Ther., 279, 1453-1461 (1996); 2) J. Pharmacol. Exp. Ther., 284, 687-692 (1998); 3) N. Engl. J. Med., 337, 1029-1035 (1997); 4) Gut., 40, 628-633 (1997) published before the priority date of the present application.

For the foregoing reasons, it is now apparent that all of the claims in this application are in condition for allowance.

No further issues remaining, allowance of this application is respectfully requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact undersigned at the telephone number below.

THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975

Respectfully submitted,

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Pharmacological Profile of SB 203580, a Selective Inhibitor of Cytokine Suppressive Binding Protein/p38 Kinase, in Animal Models of Arthritis, Bone Resorption, Endotoxin Shock and Immune Function

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ABSTRACT

SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole], a selective cytokine suppressive binding protein/p38 kinase inhibitor, was evaluated in several models of cytokine inhibition and inflammatory disease. It was demonstrated clearly to be a potent inhibitor of inflammatory cytokine production in vivo in both mice and rats with IC_{50} values of 15 to 25 mg/kg. SB 203580 possessed therapeutic activity in collagen-induced arthritis in DBA/LACJ mice with a dose of 50 mg/kg resulting in significant inhibition of paw inflammation and serum amyloid protein levels. Antiarthritic activity was also observed in adjuvant-induced arthritis in the Lewis rat when SB 203580 was administered p.o. at 30 and 60 mg/kg. Evidence for disease-modifying activity in this model was indicated by an improvement in bone mineral density and by histological eval-

uation. Additional evidence for beneficial effects on bone resorption was provided in the fetal rat long bone assay in which SB 203580 inhibited ^{46}Ca release with an IC_{50} of 0.6 μM. In keeping with the inhibitory effects on lipopolysaccharide-induced tumor necrosis factor-α in mice, SB 203580 was found to reduce mortality in a murine model of endotoxin-induced shock. In immune function studies in mice treated with SB 203580 (60 mg/kg/day for 2 weeks), there was some suppression of an antibody response to ovalbumin, whereas cellular immune functions measured ex vivo were unaffected. This novel profile of activity strongly suggests that cytokine inhibitors could provide significant benefit in the therapy of chronic inflammatory disease.

Cytokines such as IL-1 and TNF-α play a predominant role during inflammatory responses and autoimmune disease (Dinarello, 1991). Evidence for their key participation in acute and chronic inflammation has been provided by the demonstration that protein antagonists such as IL-1ra and monoclonal antibodies to TNF-α, and its soluble receptor, can interfere with various acute and chronic inflammatory responses. Another approach to the control of proinflammatory cytokines is to inhibit their production, ideally through the use of p.o. active low molecular weight compounds. One class of compounds that is effective in this respect is the pyridinyl imidazoles which have been shown to inhibit cytokine production in vitro, and in vivo they can attenuate the inflammatory components of disease in the absence of generalized

immunosuppression (Griswold et al., 1988; Lee et al., 1993; Reddy et al., 1994).

SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] (fig. 1) is a member of a new series of pyridinyl imidazole compounds which inhibit IL-1 and TNF-α production from LPS-stimulated human monocytes and the human monocyte cell line THP-1 with IC_{50} values of 50 to 100 nM (Lee et al., 1994a,b; Gallagher et al., 1995). The term CSAID™ has been coined for these compounds and they have shown activity in a number of animal models of acute and chronic inflammation (Lee et al., 1993). The molecular target of SB 203580 and related compounds has been identified as a pair of closely related mitogen-activated protein kinase homologs, alternatively termed CSBP (Lee et al., 1994b), p38 (Han et al., 1994) or RK (Rouse et al., 1994). The binding of the CSAID™ compounds to the target CSBP in
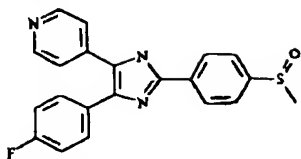
Fig. 1. Structure of SB 203580.

THP.1 cytosol correlates with their cytokine biosynthesis inhibition (Lee et al., 1994b), indicating a role for CSBP in the production of cytokines in response to various stimuli (Lee and Young, 1996).

Compounds structurally related to SB 203580 have been tested previously in a number of animal models for their anti-inflammatory activity, including collagen-induced arthritis (Griswold et al., 1988) and endotoxin shock (Badger et al., 1989; Olivera et al., 1992). These models are relatively insensitive to CO inhibitors, which adds credence to the cytokine suppressive nature of the CSAID™ molecules. In this manuscript, we show that cytokine inhibition with SB 203580 has beneficial effects in animal models of disease with only minor effects on immune function.

Materials and Methods

Animals. DBA/1 LACJ, BALB/c and C57BL/6 male mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male Lewis rats were obtained from Charles River Laboratories (Raleigh, NC.) Within any given experiment, only animals of the same age were used. All experimental procedures were in accordance with National Institutes of Health guidelines and were reviewed by the SmithKline Beecham Animal Care and Use Committee (King of Prussia, PA).

Materials. SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinyl)-phenyl)-5-(4-pyridyl)imidazole] was synthesized at SmithKline Beecham Pharmaceuticals (fig. 1). For in vivo assays, SB 203580 was administered p.o. in 0.03 N HCl-0.5% tragacanth (Sigma Chemical Co., St. Louis, MO) at the doses indicated. RAP was prepared by fermentation at SmithKline Beecham Pharmaceuticals (Brockham Park, UK). RPMI 1640 was obtained from Flow Laboratories (Rockville, MD and contained 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine (GIBCO, Grand Island, NY). This medium will be known as RPMI-10. Con A was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Endotoxin (LPS) was either Escherichia coli, type W or Salmonella typhosa (Difco Laboratories, Detroit MI) and OVA was from Sigma.

LPS-induced TNF production in mice and rats. BALB/c male mice in groups of three to five were treated with vehicle or compound by p.o. gavage and 30 min later the animals were injected i.p. with 25 μg/mouse of endotoxin (E. coli, type W, Difco). Two hours later, the animals were euthanized by carbon dioxide asphyxiation and plasma was obtained from individual animals by collecting blood into heparinized tubes. The samples were clarified by centrifugation at 12,500 × g for 5 min at 4°C. The supernatants were decanted to new tubes (may be stored at −20°C) and were assayed for mouse TNF-α by ELISA (Olivera et al., 1992). The range of sensitivity of the ELISA is 25 to 800 pg/ml of mouse TNF-α. For the induction of TNF-α in Lewis rats, the animals were treated with SB 203580 30 min before the injection of LPS (30 μg/kg i.p.). TNF-α levels were measured 90 min later by ELISA.

Collagen-induced arthritis. Type II collagen arthritis was induced in male DBA/1 LACJ mice (30–35 g, Jackson Laboratories) by the method of Wooley (1988). The mice were primed with an emulsion consisting of CFA (Difco Laboratories) combined with an equal volume of a freshly prepared solution of 2.0 mg of collagen type II (bovine nasal septum, Elastin Products Co., Inc., Owensville, MO)

per ml of 0.01 N acetic acid. Extra Mycobacterium butyricum (Difco) was added to the CFA to make the concentration twice that present in the commercial preparation. The CFA/collagen emulsion was prepared by mixing through two connected 20-ml syringes. An intradermal injection of 0.1 ml of emulsion per mouse was administered at the base of the tail. Twenty-one days later, the mice were boosted by an i.p. injection of 0.1 ml of freshly prepared 1.0 mg of bovine collagen II/ml of 0.01 N acetic acid per mouse. Joint swelling presented within a few days and the mice were evaluated for incidence and severity of inflammation, assigned randomly to study groups, ear tagged and the individual mouse's dosing regimen was begun. Severity of joint swelling was determined subjectively for each limb by using a scale of 1 (one or more phalanges per limb) to 4 (maximum swelling per limb). A severity score of at least 2 on one limb (excluding phalanges) was required for an animal to be assigned to a study group. Before dosing, each mouse was bled by the tail vein for a serum sample (100–150 μl of blood). Disease severity was assessed on days 7 and 10 after which blood was collected (tail vein on day 7 and by exsanguination on day 10) for serum. The serum samples were assayed for mouse SAP by using a radio immunologically quantitated Western blot method (Griswold et al., 1988).

Statistical analysis. Clinical severity and levels of SAP and TNF-α were analyzed by using the Student's t test, with P values less than .05 considered significant.

AA. AA was induced by a single injection of 0.75 mg of M. butyricum (Difco) suspended in paraffin oil into the base of the tail of male Lewis rats, 6 to 8 weeks old (160–180 g). Hindpaw volumes were measured by a water displacement method on day 16 and/or day 22. Test compounds were homogenized in acidified 0.5% tragacanth (Sigma) and were administered p.o. in a volume of 10 ml/kg. Control animals were administered vehicle (tragacanth) alone.

Percentage of inhibition of hindpaw lesions was calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{AA (Treated)}}{\text{AA (Normal)}} \times 100$$

For statistical analysis, paw volumes of rats treated with SB 203580 were compared to the untreated controls by Student's t test.

BMD, as well as BMC and bone area were determined for the distal tibia by DXA by using the Hologic QDR-1000 equipped with high resolution scanning software as we have described previously (Bradbeer et al., 1996).

Tibio-tarsal joints from representative animals from the following three groups of rats were examined histologically; normal rats, AA control rats and AA rats treated with SB 203580 at 60 mg/kg/day. Rats were sacrificed by CO₂ administration and the rear legs were fixed in formalin, decalcified in formic acid and the feet removed from the legs at the distal tibial diaphysis. After routine processing, the feet were embedded and coronal sections were cut in the plane midway through the tibiotarsal and tarsotarsal joints. Sections were stained with Safranin O and counterstained with fast green.

Bioassay for IL-6. Serum samples were obtained when the rats were euthanized. IL-6 levels were determined by using the previously described B9 bioassay (Aarden et al., 1985). Briefly, B9 cells (5 × 10³ cells/well in 96-well flat-bottomed plates) were cultured at 37°C with serial dilutions of rat serum in a final volume of 100 μl of RPMI-10. After 68 hr, 0.5 μCi of [³H]thymidine was added and was incubated for 6 hr at 37°C. Cells were harvested and radioactivity incorporation was determined. IL-6 was quantified from a standard curve including known amounts of rat IL-6 (0.1–100 pg/ml). B9 proliferation was unaffected by any agents used in this study.

Fetal rat long bone resorption assay. This assay was performed essentially as described previously (Raisz, 1965; Stern and Raisz, 1979). Timed-pregnant Sprague Dawley rats (Taconic Farms, Germantown, NY) were injected s.c. with 200 μCi of ⁴⁵CaCl₂ on day 18 of gestation, housed overnight, then anesthetized with Innovar-Vet (Pittman-Moore, Mundelein, IL) and sacrificed by cervical dislo-
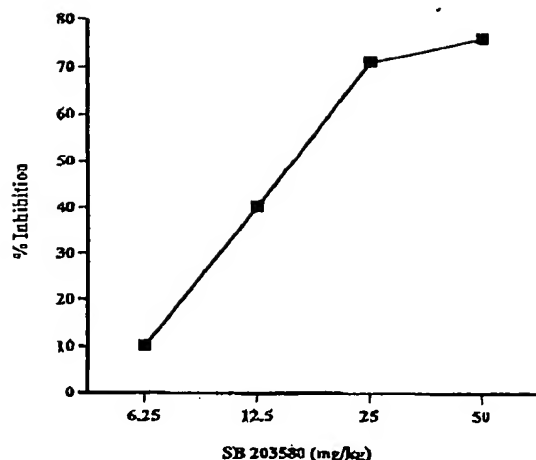
Fig. 2. Inhibition of plasma TNF levels in BALB/c mice. SB 203580 was administered 30 min before LPS challenge and TNF was measured by ELISA 2 hr later. Data are presented as percentage of inhibition by using three to five animals per group. Significant inhibition was observed at 50, 25 and 12.5 mg/kg (P < .001) with an IC$_{50}$ of 15 mg/kg.

cation. Fetuses were removed aseptically and radii and ulnae were dissected free of surrounding soft tissue and cartilaginous ends. The bones were cultured 18 to 24 hr in BGJ$_b$ medium (Sigma) containing 1 mg/ml of bovine serum albumin, then were transferred to fresh medium and cultured for an additional 48 hr in the absence or presence of 50 ng/ml of PTH (human, 1–34) and test compound. Calcium released into the medium and total residual calcium in the bones were measured by liquid scintillation spectrometry. Data are expressed as the percentage of calcium released from treated bones as compared to corresponding control bones. Statistical differences were assessed by using a one-way analysis of variance for nonpaired samples. Data are presented as mean ± S.E.

Endotoxin shock. Pathogen-free male C57BL/6 mice were obtained from Jackson Laboratories. Age-matched mice, 6 to 12 weeks old, were used. This model of shock was performed as described previously (Badger et al., 1989; Olivera et al., 1992). Briefly, 0.1 μg of LPS from Salmonella typhosa (Difco) mixed with D-(+)-gal (Sigma: 500 mg/kg) was injected i.v. in 0.25 ml of pyrogen-free saline (this mixture is referred to as LPS/D-gal). Compounds to be tested were administered p.o. 30 min before the i.v. injection of LPS/D-gal. Blood was collected via cardiac puncture 1 hr after LPS/D-gal and serum samples were stored at −20°C until evaluation for TNF-α by ELISA. Survival was monitored, in separate groups of animals, for 48 hr after LPS challenge, at which time no further deaths occurred in either treated or untreated control mice.

Immune function assays. Female BALB/c mice were immunized with 100 μg of OVA in 50 μl of CFA in both hind footpads (OVA was prepared at 4 mg/ml and diluted 1:1 in CFA). Mice were then

treated for 5 days a week for 2 weeks with 60 mg/kg of SB 203580 or 50 mg/kg of RAP administered i.p. in a vehicle composed of 10% ethanol, 10% cremophor and 80% saline. At the termination of the experiment (day 12), spleen and lymph nodes were harvested and cell suspensions were prepared by standard procedures. For the response to OVA and Con A, lymph node cells (5 × 10⁵) were established in 96-well round bottomed plates in the presence or absence of serially diluted Con A or OVA for 72 hr. For the mixed lymphocyte reaction, cells from treated BALB/c mice (1 × 10⁶) were established in 96-well flat bottomed plates along with C57BL/6-irradiated (3000 R) stimulator cells (1 × 10⁶). Cell cultures were incubated at 37°C/5% CO₂, with 0.5 μCi of [³H]thymidine added for the last 18 hr of culture. Cell-associated radioactivity was measured after collection onto glass-fiber filters by scintillation counting. For OVA-specific antibody response, sera from immunized mice were tested for activity by ELISA which has been described in detail previously (Reddy et al., 1994).

Results

Inhibition of TNF-α and collagen-induced arthritis in mice. Demonstration of the ability of SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole] to inhibit inflammatory cytokine production in vivo was accomplished by using BALB/c mice challenged with LPS (25 μg i.p.). As seen in figure 2, SB 203580 given p.o. 30 min before LPS challenge inhibited the production of TNF-α (ED$_{50}$, 15 mg/kg p.o.).

Given the potent ability of SB 203580 to inhibit TNF-α production in vivo, it was of interest to evaluate the effect of the compound on a chronic inflammatory model. Collagen-induced arthritis was induced in DBA/1 LACJ mice by injection of bovine Type II collagen in CFA at the base of the tail, followed 21 days later by a booster injection of collagen solubilized in acetic acid (i.p.). Animals with significant disease were treated with SB 203580 (50 mg/kg p.o., b.i.d.). At the end of 7 days, the disease severity was judged on a scale of 0 to 4⁺ and blood was obtained for analysis of serum amyloid P component. As seen in table 1, in two separate studies, SB 203580 significantly reduced disease severity (72%, P < .01 and 45%, P < .05, respectively) as well as acute phase reactant (SAP) levels (42%, P < .05 and 52%, P < .001, respectively).

Inhibition of TNF-α and AA in rats. TNF-α was also inhibited in SB 203580-treated Lewis rats. This was shown by treating normal rats with SB 203580 p.o. 30 min before a challenge with 30 mg/kg of LPS i.p. Plasma TNF-α levels measured 90 min later were inhibited by 53% at 25 mg/kg (P < .01) and by 38% at 12.5 mg/kg (P < .01) with no inhibition observed at 6.2 mg/kg (table 2).

In the rat model of AA, p.o. administration of SB 203580

TABLE 1

Effect of SB 203580 on type II collagen-induced Arthritis in DBA/1 mice

This table summarizes two studies in which the mice were dosed for 7 days at 50 mg/kg (p.o., b.i.d.), after the animals had presented with paw or joint edema/swelling. The data are the mean ± S.E. from a group of vehicle (0.03 N HCl/0.5% Tragacanth) and SB 203580-treated controls. In Experiment II, the mice were entered into the study as fully complemented groups. Data are significantly different from the control: * P < .05; ** P < .01; *** P < .001.

Treatment	n	Severity Index	%	n	SAP	%
Experiment I						
Control	9	5.86 ± 1.17		9	189.89 ± 28.29	
SB 203580	8	1.63 ± 0.79	72**	8	110.66 ± 17.75	42*
Experiment II						
Control	10	5.75 ± 0.63		8	181.30 ± 10.36	
SB 203580	10	3.15 ± 0.51	45*	8	86.61 ± 6.23	52***

TABLE 2

Inhibition of LPS-stimulated TNF-α levels in SB 203580 Lewis rats

Rats were dosed (p.o.) 30 min before treatment with LPS (30 μg/kg/i.p.). Plasma TNF was measured 90 min after LPS administration. Data are mean ± S.E. for six animals per group. * P < .05; ** P < .01.

Treatment	TNF-α	% Inhibition
	ng/ml	
Control (untreated)	42.15 ± 5.05	
SB 203580		
25 mg/kg	19.91 ± 2.77	53**
12.5 mg/kg	26.20 ± 4.56	38*
6.25 mg/kg	36.76 ± 3.56	13 N.S.

(10, 30 and 60 mg/kg p.o.) from day 0 to day 22 inhibited the development of immune-mediated hindpaw inflammation. On day 16, there was 86% inhibition at 60 mg/kg (P < .001) and 62% inhibition at 30 mg/kg (P < .01), with no effect observed at 10 mg/kg (fig. 3A). By day 22, the anti-inflammatory effect had lessened somewhat with 60% (P < .001) and 45% (P < .01) inhibition at 60 and 30 mg/kg, respectively (fig. 3B).

The anti-inflammatory and antiarthritic activities of SB

203580 were evaluated further by examining the BMC and BMD of the distal tibia region in treated AA rats. On day 22 when the rats were euthanized, hindlimbs were examined by DXA. When compared with the AA controls, there was a significant normalization of BMD (31%, P < .01) and BMC (26%, P < .01) in the rats treated with 60 mg/kg/day of the compound, indicating a protective effect on inflammation-mediated bone destruction and/or a direct effect on bone resorption proximal to the inflamed joint (fig. 4, A and B).

Histology of the tibio-tarsal joint from a normal rat and from rats challenged with adjuvant and then treated with vehicle (AA control) or SB 203580 is shown in figure 5. In the AA control joint, all of the original bone and marrow has been replaced by granulation tissue and newly formed woven bone. Remnants of articular cartilage are evident and the
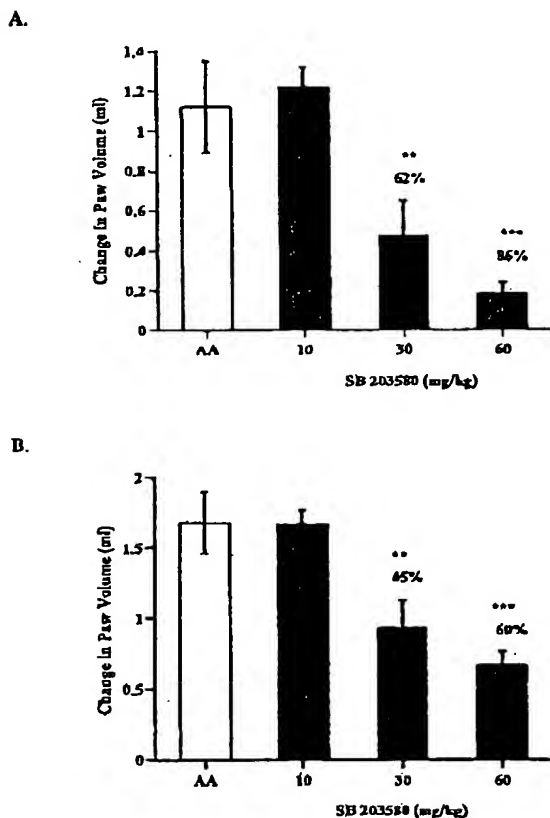
A.

A.

B.

B.

Fig. 3. Dose-dependent suppression of hindpaw inflammation in rats with AA by prophylactic administration of SB 203580 from days 0 to 22 (5 days a week). Paw inflammation was measured on day 16 (A) and on day 22 (B). Data are the mean and S.E.M. of 10 animals per group. **P < .01; ***P < .001, compared to the untreated AA controls.
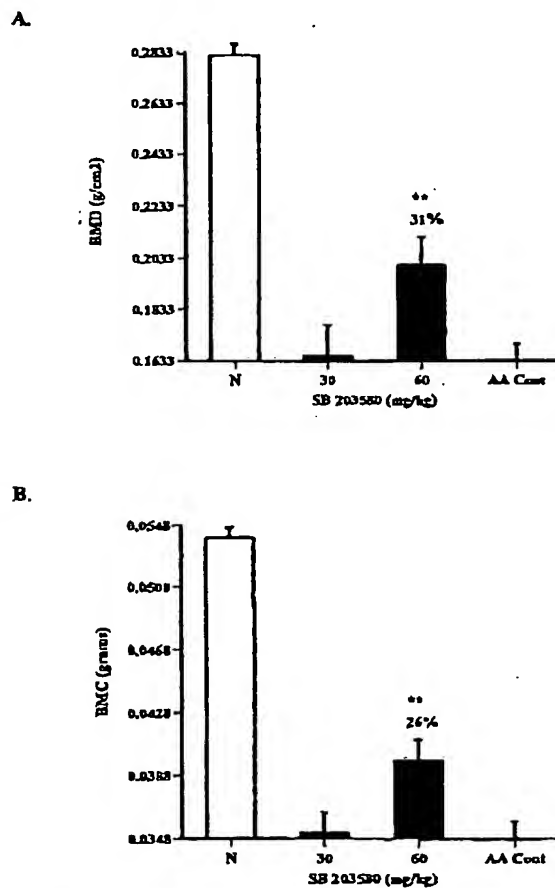
Fig. 4. Bone densitometry evaluation of the distal tibia in AA rats treated with SB 203580. Rats were treated with various doses 5 days a week from day 0 to day 22. Values are the percentage of normal (assigned a value of 100%), mean and S.E.M. of 10 animals per group. A, the BMD value was 0.2822 ± 0.0045 for normal rats and 0.01833 ± 0.0067 for AA rats, which is a 42% decrease in BMD in the diseased animals. B, the BMC value was 0.0540 ± 0.0007 for normal rats and 0.0348 ± 0.001 for AA rats which is a 36% decrease in BMC in the diseased animals. The effect of SB 203580 was statistically significant on both BMD and BMC. **P < .01.
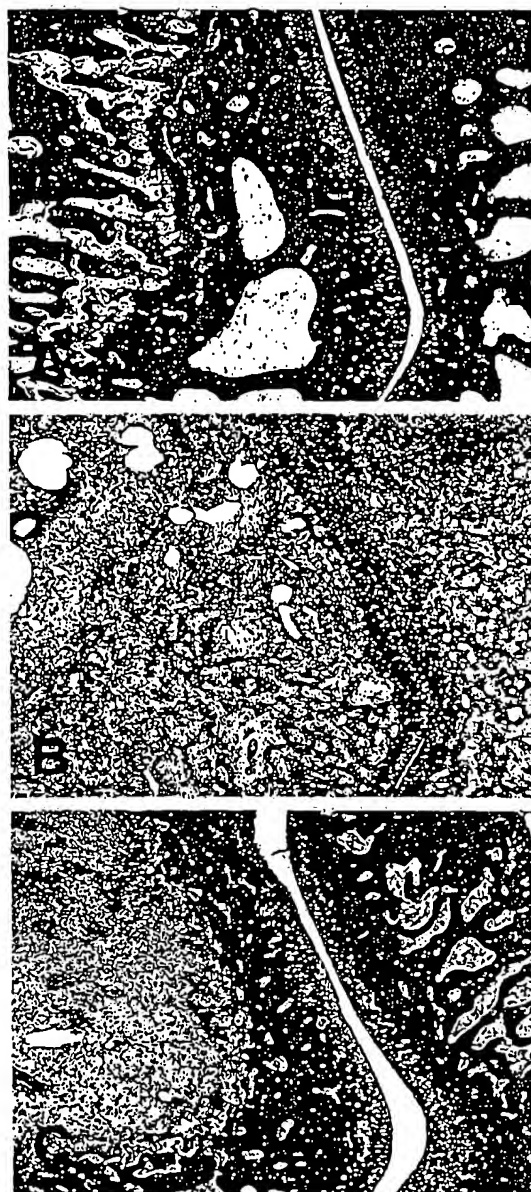
Fig. 5. A, photomicrograph of a normal tibio-tarsal joint. Cancellous bone of the metaphysis (left) is separated from the epiphysis (middle) by the growth plate (continuous pink line). The joint space between the tibia and tarsus (right) is clearly visible. B, photomicrograph of a tibio-tarsal joint typical of a rat suffering from AA. All of the original normal marrow and bone of the tibia and tarsus has been replaced by granulation tissue within which reactive woven bone has formed. The joint space (running vertically in approximately the same location as in the upper and lower panels) has been obliterated almost entirely by infiltrating granulation tissue and a large region of the articular cartilage on the tarsal side of the joint has been replaced by granulation tissue. C, photomicrograph of a tibio-tarsal joint from a rat which had been challenged with adjuvant and then treated with SB 203580 (60 mg/kg/day) for 22 days. Although a small amount of infiltration and cartilage

TABLE 3

Inhibition of serum IL-6 in AA rats treated with SB 203580

SB 203580 was administered orally 5X a week from day 0 to 22. Serum IL-6 was measured on day 23. Data are mean ± S.E. for 10 animals per group. *** P < .001.

Treatment	IL-6	% Inhibition
	ng/ml	
Control AA rats	1.85 ± 0.10	
SB 203580		
60 mg/kg	1.11 ± 0.13	40***
30 mg/kg	1.43 ± 0.10	23***
10 mg/kg	1.75 ± 0.07	5 N.S.

former joint space has been infiltrated with granulation tissue. The joint from the rat treated with SB 203580 shows protection of the joint space, articular surfaces and subchondral bone. Although the tibial metaphyseal cancellous bone and marrow have been replaced by granulation tissue, these components of the tarsus are normal. This histological appearance is consistent with SB 203580 having retarded the progression of the adjuvant-induced arthritic lesion.

Serum IL-6 levels in AA rats treated with SB 203580 on days 0 to 22 were measured in a B9 hybridoma proliferation assay. Normal rats had serum IL-6 levels of < 50 pg/ml, whereas levels in rats with untreated AA were elevated as high as 1.85 ng/ml. In rats treated with SB 203580, there was a 40% reduction in IL-6 at the 60 mg/kg dose (P < .001) and 23% inhibition at 30 mg/kg (P < .001) (table 3).

Fetal rat long bone assay. As studies in the AA rat showed clearly that treatment with SB 203580 had disease-modifying activity and protective effects on both bone and cartilage, we examined the effect of the compound in a fetal rat long bone resorption assay. In this assay, osteoclast-mediated bone resorption is monitored by measuring the release of ^{45}Ca into the culture medium from preradiolabeled fetal long bones. SB 203580 inhibited resorption in a concentration-dependent manner; 3 µM (85%, P < .001), 1 µM (80%, P < .001) and 0.3 µM (38%, P < .05). The IC_{50} was 0.6 µM (fig. 6).

Endotoxin shock. The effect of SB 203580 was evaluated in a mouse model of endotoxin shock. In this model, C57BL/6 mice are sensitized with D-(+)-gal, which makes them highly susceptible to the lethal effects of endotoxin (LPS). One hour before an i.v. injection of LPS/D-gal, control mice have serum levels of TNF-α up to 4 ng/ml. This is reduced in a dose-dependent manner by prophylactic administration of SB 203580 given 30 min before the injection of LPS/D-gal. Doses of 100, 50 and 25 mg/kg were active and inhibited TNF-α levels by 87% (P < .001), 62% (P < .001) and 42% (P < .001), respectively (table 4). In a separate group of mice that were monitored for survival, 84% of mice treated with the 100 mg/kg dose of SB 203580 survived compared to only 17% of control mice.

Immune function. In order to determine whether chronic administration of a CSAID™ molecule such as SB 203580 had detrimental (suppressive) effects on the immune system, BALB/c mice were immunized with OVA in CFA and then treated for 2 weeks (5 days a week) with 60 mg/kg i.p. of the

erosion can be seen on the tibial side (top), the joint space and articular surface are otherwise normal. Whereas the cancellous bone of the tibial metaphysis has been lost and the marrow largely replaced by granulation tissue, woven bone has not yet formed. The bone and marrow of the tibial epiphysis and the tarsus are normal.
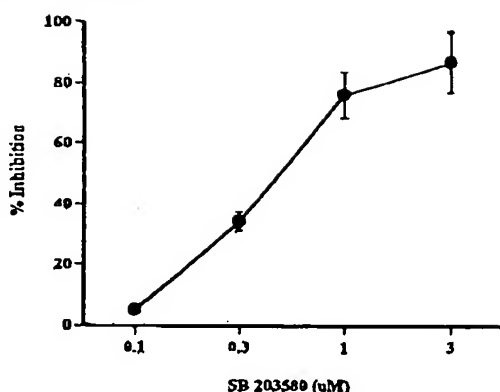
Fig. 6. SB 203580 inhibits/PTH-stimulated fetal rat long bone resorption *in vitro* ($IC_{50} \approx 0.6\ \mu M$). Fetal rat radii and ulnae (four bones per treatment per experiment) were cultured in the presence of 50 ng/ml of PTH and the indicated concentrations of SB 203580 for 48 hr as described under "Materials and Methods." Each data point represents mean and S.E.M. from three separate experiments. Bones cultured in the absence of PTH released approximately 14% of the incorporated ^{45}Ca. In the presence of PTH, control bones released approximately 45% of the incorporated ^{45}Ca.

compound or with RAP at 50 mg/kg. The serum antibody response of mice treated with RAP was suppressed totally by this treatment and there was a significant reduction in the anti-OVA serum antibody titer in mice treated with SB 203580 (fig. 7A). However, when lymph node cells from treated mice were examined for their response to the specific OVA antigen or to the mitogen Con A, no inhibition of proliferation was observed (fig. 7, B and C). Neither was there any inhibition of an allogeneic response in a mixed lymphocyte reaction between spleen cells from treated mice and C57BL/6 stimulator (3000 R) cells (fig. 7D). In all cases, the lymphocyte responses of RAP-treated mice were suppressed dramatically.

Discussion

The pyridinyl imidazoles are a novel class of compounds that have potent inhibitory effects on cytokine production both *in vitro* and *in vivo*, and also show anti-inflammatory activity in a variety of animal models (reviewed in Lee *et al.*, 1993). An early compound in this series, SK&F 86002, had cytokine suppressive activity (IC_{50}, 1 μM) (Lee *et al.*, 1993), but no significant antiproliferative activity (Reddy *et al.*, 1994). In addition to cytokine suppressive activity, SK&F 86002 and many structurally related analogs inhibited eico-

sanoid metabolism in LO and CO enzyme assays (Griswold *et al.*, 1987). In keeping with this profile of both cytokine and eicosanoid inhibition, SK&F 86002 and related compounds showed therapeutic activity in mouse collagen-induced arthritis (Griswold *et al.*, 1988) and carageenan-induced inflammation (Lee *et al.*, 1993), as well as analgesic activity in mouse abdominal constriction assays (Lee *et al.*, 1993). These activities, however, could not totally be attributed to LO/CO inhibition and the compounds clearly did not act as classical nonsteroidal anti-inflammatory drugs. Evidence for this was their activity in assays and models relatively insensitive to CO inhibition such as collagen-induced arthritis (Griswold *et al.*, 1988), the fetal rat long bone resorption assay (Votta and Bertolini, 1994) and mouse models of endotoxin shock (Badger *et al.*, 1989; Olivera *et al.*, 1992).

In studies designed to define the mechanism of cytokine suppression by the pyridinyl imidazoles, it was revealed that inhibition of TNF-α synthesis was primarily at the translational rather than the transcriptional level (Lee *et al.*, 1990; Young *et al.*, 1993), and that a block occurred before nascent peptide elongation (Young *et al.*, 1993; Prichett *et al.*, 1995; P. R. Young, unpublished data). Recent investigations using THP.1 cells, radiolabeled chemical probes for radioligand binding assays and photoaffinity labeling experiments have identified the molecular target of these compounds to be a pair of closely related mitogen-activated protein kinase homologs termed CSBPs (Lee *et al.*, 1994b). CSBP, alternatively termed p38 or RK, has subsequently been identified independently by several laboratories (Lee *et al.*, 1994b; Han *et al.*, 1994; Rouse *et al.*, 1994).

Inhibition of CSBP kinase activity by these compounds correlates with cytokine inhibition and THP.1 cytosol binding assays (Lee *et al.*, 1994b). SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole], a newer member of the pyridinyl imidazoles, is the best studied compound and has an IC_{50} of 0.22 μM as a CSBP inhibitor (Cuenda *et al.*, 1995; Gallagher *et al.*, 1995; T. F. Gallagher *et al.*, in press, 1996). The compound is highly specific for CSBP kinase with no inhibitory activity observed on a variety of other kinases (Cuenda *et al.*, 1995). A physiological substrate of CSBP is MAPKAP kinase-2, and SB 203580 inhibits the activation of this kinase and its subsequent phosphorylation of hsp 27 in stress-stimulated cells (Cuenda *et al.*, 1995). In *in vitro* monocyte cultures, SB 203580 inhibits IL-1 and TNF-α from LPS-stimulated human monocytes (IC_{50}, 50–100 nM) as well as the production of leukotriene B_4 from calcium ionophore (A23187)-stimulated human monocytes (IC_{50}, 1.5 μM) (M. D. Chabot-Fletcher, unpublished observations). In HL-60 cells, SB 203580 had little effect on the LO pathway,

TABLE 4

SB 203580 inhibits serum TNFα and improves survival in a murine model of endotoxin shock

Male C57BL/6 mice were treated p.o. with SB 203580, 30 min before LPS/b-gal given i.v. Serum TNF was measured 1 hr later. Data are mean and S.E.M. of livas to five animals per group. Survival was monitored in a separate group of mice (six per group). * P < .05 by Fisher's exact test. ND = not done.

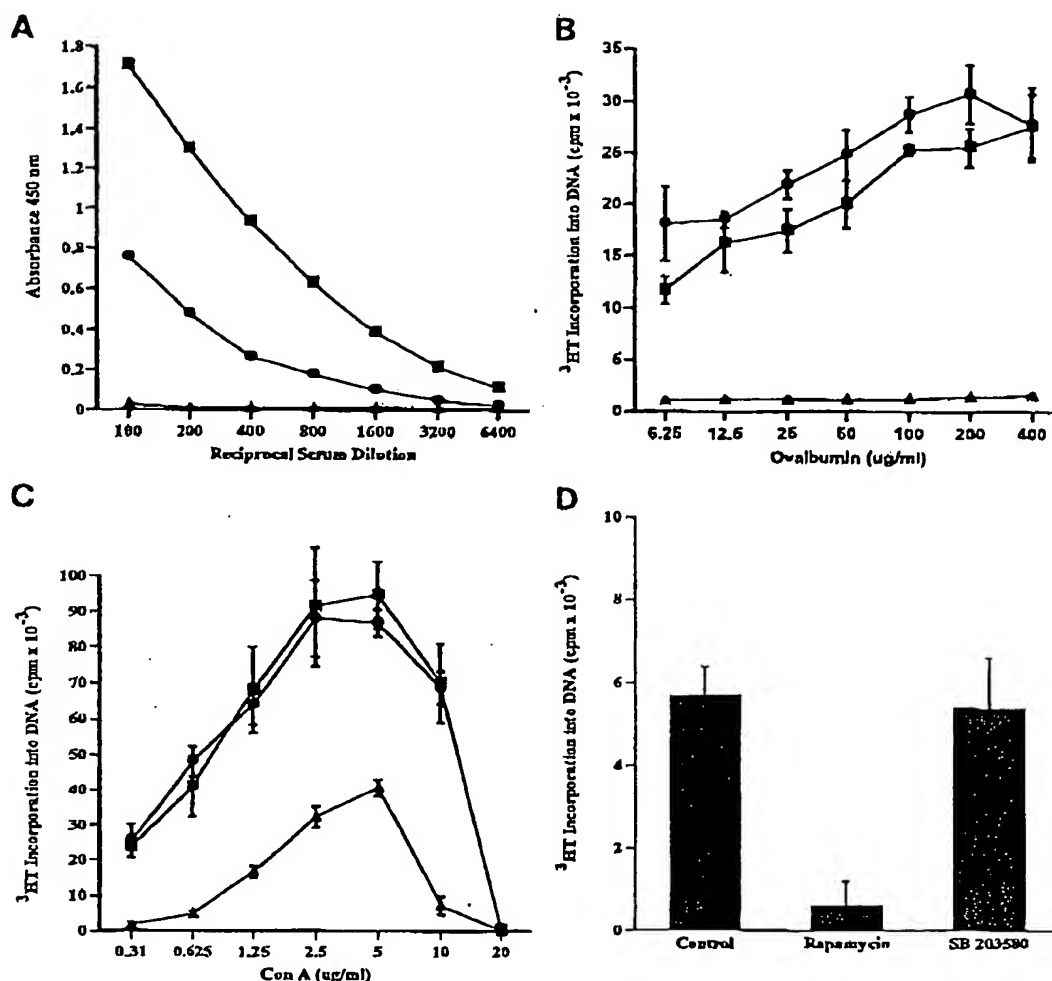| Treatment/Dose	TNF-α	% Inhibition	% Survival
	pg/ml		
Control	3789 ± 142		17
SB 203580			
100 mg/kg	490 ± 172	87**	84*
50 mg/kg	1444 ± 130	62**	20 N.S.
25 mg/kg	2248 ± 130	42**	ND
12.5 mg/kg	3514 ± 214	7 N.S.	ND

Fig. 7. Immune function in BALB/c mice treated with SB 203580 at 60 mg/kg i.p. for 2 weeks. Mice were immunized in the hind footpads with OVA in CFA as described under "Materials and Methods." A, serum antibody response to OVA measured in an ELISA assay. Data are from pooled serum samples. B, proliferative response of lymph node cells to varying doses of OVA. Data are mean and S.D. of six replicates. C, proliferative response of lymph node cells to varying doses of Con A. Data are mean and S.D. of six replicates. ■, control untreated; ●, SB 203580; ▲, RAP; and D, mixed lymphocyte reaction of treated BALB/c spleen cells against irradiated (3000 R) C57BL/6 stimulator cells. Data are mean and S.D. of six replicates.

but potently inhibited prostaglandin E_2 synthesis. SB 203580, however, had no direct inhibitory activity on pituitary growth hormone Synthase-1 and only modest inhibitory activity on LO (IC_{50}, 58 μM). The involvement of CSBP in the regulation of arachidonic acid availability, which is the rate limiting step for both LO and CO production, provides one mechanism which may explain these observations. In platelets, CSBP mediates the activation of cytosolic phospholipase A_2 by phosphorylation of cytosolic phospholipase A_2 in response to a thrombin agonist peptide (Kramer et al., 1995). This activation was correlated with the subsequent release of arachidonic acid and formation of CO products. The observation that SB 203580 inhibits the synthesis of the inducible COX-2 enzyme provides an additional mechanism by which CSBP can regulate prostanoid synthesis (Lee et al., 1994a).

In the studies reported in this paper, we have profiled SB 203580 in a number of pharmacological models both in vitro and in vivo and demonstrated its activity in a wide variety of TNF-α-mediated animal models. SB 203580 inhibited LPS-induced TNF-α in vivo in both mice and rats with IC_{50} values of 15 and 25 mg/kg, respectively. This inhibition of TNF-α was an indication that disease models such as mouse collagen-induced arthritis and rat adjuvant arthritis would be positively modulated by the compound. This was indeed the case and, in collagen-induced disease in DBA/1 LACJ mice, SB 203580 dosed for 7 days at 50 mg/kg p.o. (b.i.d.) reduced joint edema by 72 and 45% in two separate experiments. SAP, an acute inflammatory protein in mice, was also inhibited by 42 and 52%, respectively, in the two experiments. Evidence for the critical role of endogenous TNF-α in this disease model

has been provided by the observations that administration of anti-TNF-α antibodies can ameliorate the disease (Piguet et al., 1992; Thorbecke et al., 1992; Williams et al., 1992) and that TNF-α transgenic mice spontaneously develop arthritis (Keffer et al., 1991).

TNF-α clearly plays a proinflammatory role in another animal model of RA, the AA rat, in which elevated levels have been observed in the plasma and joints (DiMartino et al., 1993; Smith-Oliver et al., 1993). In this disease model, SB 203580 was very effective in reducing paw inflammation at doses of 30 and 60 mg/kg/day with optimum inhibition observed at 60 mg/kg/day (86% inhibition on day 16). Evidence for the protection of joint integrity at this dose was provided by the observation that there was a normalization of BMD (31%) and BMC (26%) as measured by DXA. This was also reflected in the histological evaluation of the affected joints, in which a clear beneficial effect was observed on both bone and cartilage. In keeping with the compound's disease-modifying activity, our studies also demonstrated that serum levels of IL-6 were reduced in treated rats. This cytokine has been shown to be increased in different biological fluids in patients with autoimmune disease, particularly RA (Houssiau et al., 1988; Swaak et al., 1988; Hirano et al., 1988), and the level in various inflammatory compartments appears to be a sensitive marker of disease activity.

The protection of bone integrity in the AA rat led us to evaluate SB 203580 in a direct in vitro assay of bone resorption, the fetal rat long bone assay. Cytokines such as IL-1 and TNF-α have been shown to stimulate bone resorption in vitro and in vivo (Gowen and Mundy, 1986; Bertolini et al., 1986; Tashijian et al., 1987; Sabatini et al., 1988), and it was reasonable to expect that a CSAID™ molecule would have a protective effect in this model system. SB 203580 dose-dependently (IC_{50}, 0.6 μM) inhibited PTH-stimulated bone resorption. Although the precise mechanism of action of the compound (and other pyridinyl imidazoles) on bone resorption has not been defined fully, it appears to be related to the compound's cytokine suppressive properties as selective CO, and dual CO/LO inhibitors were inactive in this organ culture system (Votta and Bertolini, 1994).

Another animal model in which TNF-α has been shown to play a predominant role is that of endotoxin-induced shock. We demonstrated previously that SK&F 86002, a dual inhibitor of arachidonic acid metabolism as well as a cytokine inhibitor, could reduce serum TNF-α levels and prolong survival in mouse shock models (Badger et al., 1988). In addition, we were able to demonstrate that antibodies to mouse TNF-α could protect mice against endotoxin-induced shock in mice that were sensitized with Proprionibacterium acnes (Badger et al., 1989). SB 203580, a more selective cytokine inhibitor with reduced inhibitory activity on LO and CO, reduced serum TNF-α in LPS/D-gal-sensitized mice and improved their survival at high doses.

It is clear that SB 203580 is a potent inhibitor of IL-1 and TNF-α in vitro and that it is pharmacologically active in a number of animal models in vivo. The question of whether such a potent cytokine inhibitor would be immunosuppressive as well as having anti-inflammatory activity has been addressed by examining its activity in vivo in mice immunized with OVA. Apart from partial inhibition of specific antibody levels against OVA, there was no suppression of OVA-specific T-cell proliferation, an allogeneic response or of

mitogen (Con A)-induced proliferative responses. These results and those reported previously with the dual inhibitor of arachidonic acid metabolism, SK&F 86002 (Lee et al., 1993; Reddy et al., 1994), show clearly that these compounds do not have overt immunosuppressive activity.

Mechanistically, it is not clear at the present time to what extent the beneficial effects of SB 203580 are due to suppression of TNF-α production or suppression of cytokine signaling. Given that SB 203580 has been shown, at least in vitro, to be effective in inhibiting cytokine signaling leading to either cytokine production or other downstream effects, it is safe to assume that in vivo, the compound may induce its antiarthritic activity via both the inhibition of cytokine production and action. As an in vitro example, SB 203580 has been shown to block IL-6 production in L929 cells stimulated with TNF-α (Beyaert et al., 1996).

The pharmacological profile that we have described here for SB 203580, a potent CSBP/p38 kinase inhibitor, would appear to be one that would be desirable for an antiarthritic therapeutic agent. Despite numerous attempts over the years to design drugs with therapeutic potential for RA, there is still a real need for more effective, less toxic treatments to control the progression of this disease. Most therapies, although supplying symptomatic relief, do not alter the progression of bone and cartilage destruction in the affected joints. In recent years, it has become clear that a multitude of cytokines contribute to the overall inflammatory and bone destructive sequelae that occur in RA, and that targeting one or more of these cytokines could modulate the disease (Arend and Dayer, 1965; Elliott and Maini, 1995). TNF-α has emerged as a cytokine of pivotal importance in the disease process and inhibition of the production and/or effects of this cytokine is a rational therapeutic strategy (Feldmann et al., 1994; Brennan et al., 1995). Indeed, ongoing studies are demonstrating the efficacy of treatment of RA with monoclonal antibodies to TNF-α in RA patients (Elliott et al., 1993; Maini et al., 1995). A small molecular weight orally active cytokine inhibitor with the pharmacological profile described in this manuscript could well provide significant beneficial effects in this disease.

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Pharmacological Effects of SB 220025, a Selective Inhibitor of P38 Mitogen-Activated Protein Kinase, in Angiogenesis and Chronic Inflammatory Disease Models

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ABSTRACT

Chronic inflammatory diseases often are accompanied by intense angiogenesis, supporting the destructive proliferation of inflammatory tissues. A model of inflammatory angiogenesis is the murine air pouch granuloma, which has a hyperangiogenic component. In this model, we explored the regulation of inflammatory angiogenesis using SB 220025, a specific inhibitor of human p38 mitogen-activated protein (MAP) kinase, with an IC_{50} value of 60 nM and 50- to 1000-fold selectivity *vs.* other kinases tested. *In vivo*, this compound reduced the lipopolysaccharide-induced production of tumor necrosis factor at an ED_{50} value of 7.5 mg/kg. In the inflammatory angiogenesis model, over the course of granuloma development, we observed elevated levels of interleukin-1β and tumor necrosis factor-α during the chronic inflammatory phase when intense angiogenesis occurs. SB 220025 at 30 mg/kg b.i.d. p.o. was able to greatly reduce the expression of these cytokines and inhibit angiogenesis by ≈40%. To further study the effects of p38/CSBP MAP kinase inhibition in angiogenesis-dependent chronic inflammatory disease, SB 220025 was tested in murine collagen-induced arthritis. In this model, SB 220025 was able to prevent the progression of established arthritis. Thus, this p38/CSBP MAP kinase inhibitor, which can reduce inflammatory cytokine production and inhibit angiogenesis, is an effective treatment for chronic proliferative inflammatory disease.

Proliferating tissues require angiogenesis to support their growth, and thus diseases such as cancer and chronic inflammation are thought to be angiogenesis dependent (Folkman, 1995; Jackson, 1996). In the case of chronic inflammation, angiogenesis may be required not only to support the proliferation but also to allow the massive cellular infiltration associated with the chronically inflamed state. Angiogenesis is normally under very tight control. In the normal adult, the majority of the vasculature is stable, with endothelial cell turnover on the order of thousands of days. Nevertheless, these quiescent cells can rapidly switch to an angiogenic phenotype under certain conditions, as, for example, in wound healing. Once a new capillary bed is established, however, the endothelium normally returns to its quiescent state.

Understanding the signals that regulate angiogenesis is key to controlling it under pathological conditions. Growth factors such as VEGF and FGF are clearly able to induce angiogenesis and, in the case of VEGF, appear to be regulated by physiological signals like hypoxia. Hypoxia is not always necessary, however; some inflammatory mediators can potently induce angiogenesis *in vivo* even in the absence of hypoxia. Both IL-1β and TNF-α can induce angiogenesis in the normally avascular cornea (BenEzra *et al.*, 1990; BenEzra and Maftzir, 1996; Fajardo *et al.*, 1992). These two cytokines have numerous activities, including upregulation of other cytokines, such as IL-8; upregulation of adhesion molecule expression; stimulation of matrix metalloproteinase expression; and increased prostaglandin production (Dinarello, 1991). Many of these activities may contribute to the angiogenic activity of these cytokines. Thus, inhibition of the activity of IL-1β and TNF-α could have an obvious benefit in angiogenesis-dependent inflammatory diseases. One means of inhibiting IL-1β and TNF-α activity is by decreasing their production. SB 220025 is a new compound belonging to the CSAID™ class of cytokine biosynthesis inhibitors (Cuenda *et al.*, 1995; Lee *et al.*, 1994), which act specifically on p38/CSBP MAP kinase to block a cascade, resulting in decreased production of IL-1β and TNF-α as well as other mediators, such as IL-6 and prostaglandins (Beyaert *et al.*, 1996; Pouliot *et*

ABBREVIATIONS: CSBP, CSAID™ binding protein; EGFR, epidermal growth factor receptor; Erk, extracellular regulated kinase; FGF, fibroblast growth factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; MAP, mitogen-activated protein; PK, protein kinase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

al., 1997). We used this compound to examine the role of these inflammatory mediators in inflammatory angiogenesis.

Materials and Methods

Compounds. Medroxyprogesterone was obtained from Sigma Chemical (St. Louis, MO). SB 203580 [4-(4-flourophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole)] and SB 220025 [5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole)] were synthesized by Medicinal Chemistry, SmithKline Beecham.

Measurements of kinase activity. p38 was prepared and assayed as previously described (Young *et al.*, 1997), with the following modifications: 30-μl reactions contained 25 mM HEPES, pH 7.5, 8 mM MgCL$_2$, 70 nM enzyme, 0.17 mM ATP (2 μCi of ^{32}P), 0.2 mM Na$_3$VO$_4$, 0.67 mM T669 peptide (derived from the EGFR KRELVE-PLTPSGEAPNQALLR), 0.167% dimethylsulfoxide and compounds. Reactions were incubated at 30°C for 10 min, stopped with 10 μl of 0.3 M phosphoric acid, captured on phosphocellulose filter paper (P81), washed with 75 mM phosphoric acid and counted by liquid scintillation.

p56-Lck autophosphorylation assay was carried out in immune complex kinase assay as described previously (Juszczak *et al.*, 1991). EGFR autophosphorylation activity was determined in an immune complex assay as for p56-Lck (above), using anti-human EGFR antibody (Upstate Biotechnology, Lake Placid, NY) and lysates of A431 cells.

PKC was assayed using rat brain cytosol (containing all PKC isoforms)and a peptide substrate (PLSRTLSVAAKK) derived from glycogen synthase. The 50-μl reactions contained 10 mM Tris, pH 7.5, 10 mM MgCl$_2$, 0.9 mM EGTA, 1.1 mM CaCl$_2$, 40 μg/ml L-α-phosphatidyl-L-serine, 1 μg/ml 1,3-diolein, 50 μM ATP (with 0.5 μCi of ^{32}P) and 5 μg of peptide substrate. Incubation was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

PKA was assayed using the catalytic subunit of PKA (Sigma) and histone H2A as substrate. Reactions of 50 μl contained 50 mM 3-(N-morpholino)propanesulfonic acid, pH 6.5, 10 mM MgCl$_2$, 50 μM ATP (0.5 μCi of ^{32}P), 0.1 mg/ml histone and 1 μM cAMP. Incubation was for 20 min at 37°C, and the phosphorylated peptide was isolated on phosphocellulose (p81) and counted as described above.

rErk (p42/44) (Upstate Biotechnology) was assayed using T669 peptide (above) in the same reaction conditions used for p38.

LPS induction of TNF-α. The method of Olivera *et al.* (1992) was used for LPS induction of TNF-α. Balb/C mice (Charles River Labs, Wilmington, MA) were administered the test compound or vehicle (acidified tragacanth) 30 min before challenge with intraperitoneal injection of 25 μg of LPS (*Escherichia coli*, type W, 055:B5; Difco, Detroit, MI). After 120 min, blood was collected through exsanguination, and serum samples were used to measure TNF-α levels by ELISA (see below).

Murine air pouch granuloma. This model is based on the methods of Colville-Nash *et al.* (1995). Female Balb/C mice (20 ± 2 g) were used, and granulomatous tissue was induced in anesthetized animals (Aerrane; Hanna Pharmaceutical Supply, Wilmington, DE) through the injection of 3 ml of air into the dorsal subcutaneous tissue on day −1, followed by the injection of 0.5 ml of 0.1% v/v croton oil (Sigma) in Freund's complete adjuvant (Sigma) on day 0. The dosing regimen was started on day 0 and continued until day 5, in conscious animals, with the compound being solubilized in 0.2 ml of N,N,dimethyl acetoacetamide (Sigma)/Cremephor El (Sigma)/saline or water (10:10:80) (saline was used for intraperitoneal injections, whereas water was used for oral dosing). On the indicated days, the animals were anesthetized and warmed to 40°C for peripheral vasodilation. A vascular cast was made by the intravenous injection of 1 ml of solution of 10% carmine red/5% gelatin solution (Sigma). The animals were chilled at 2° to 4°C for 3 hr before the removal of the granulomatous tissue.

The removed tissue was weighed, dried at 40°C for 3 days before digestion in 0.9 ml of a 0.05 M phosphate buffer, pH 7.0, containing 12 U/ml papain (Sigma) and 0.33 g/liter N-acetyl-L-cysteine (Sigma) for 2 days at 56°C and solubilization of the carmine red with 0.1 ml of 5 mM NaOH. Samples were filtered (0.2 μm), and the carmine content was determined against a carmine standard curve read at 490 nm. Sample and standard values were determined using Delta-Soft ELISA analysis software (Biomettalics, Princeton, NJ). The vascular index is the ratio of the milligram of carmine dye per gram of dry tissue. Cedarwood oil clearing was done as described by Colville-Nash *et al.* (1995), using dissected granulomas containing a carmine vascular cast. These were fixed in ethanol and then incubated in cedarwood oil (Sigma) for >14 days, causing the tissue to become translucent and allowing the vasculature to be easily visualized *via* the carmine dye trapped within the vessels.

Cytokine ELISAs. Tissue extracts were made through homogenization of granulomas in 0.5 ml of 5 mM KH$_2$PO$_4$/0.1 g of wet tissue. IL-1β levels were determined using a Cytoscreen Immunoassay Kit (BioSource International, Camarillo, CA). TNF-α levels were determined using the following assay: plates were coated with hamster anti-murine TNF-α antibody (Genzyme, Cambridge, MA) for 2 hr at 37°C and washed and blocked with a casein/BSA solution (5 g/liter for each) for 1 hr at 37°C, and the samples were added and incubated at 4°C overnight. Plates were washed, and the secondary antibody, rabbit anti-mouse TNF-α (Genzyme), was added for 2 hr at 37°C; the plates were washed, and the tertiary antibody goat anti-rabbit peroxidase conjugate (BioSource International, Camarillo, CA) was added for 2 hr at 37°C. The plates were then washed, and substrate OPD (Sigma) was added for 20 min at room temperature. The reaction was terminated with 25 μl of 0.1 M NaF/well, the absorbance read at 460 nm. Sample values for both ELISAs are calculated using DeltaSoft ELISA analysis software (Biomettalics Inc., Princeton, NJ).

Collagen-induced arthritis. The model was described previously (Griswold *et al.*, 1988). Briefly, male DBA/1 LacJ mice (16–18 g; Jackson Labs, Bar Harbor, ME) were primed intradermally with 0.1 ml of an emulsion consisting of equal volumes of Freund's complete adjuvant and bovine type II collagen at 2 mg/ml in 0.01 N acetic acid. After 21 days, the mice were given an intraperitoneal booster of 0.1 ml of 1 mg/ml bovine type II collagen in 0.01 N acetic acid without adjuvant. After the booster, mice were evaluated daily for incidence and severity of arthritis in their limbs. Scores of 0 to 4 for each limb were determined subjectively, in which 0 equals noninvolved and 4 equals the greatest severity of erythema and swelling. The maximum possible score for an arthritic mouse was 16 (4 points/limb). When the severity score reached ≈4, which was typically within 2 weeks of the collagen booster, mice were randomly assigned to vehicle or drug-treated groups, and oral dosing with SB 220025 or vehicle alone began. This day was designated day 0, and dosing continued for 10 days. Mice were evaluated, with no attention paid to their group, on days 7 and 10. All scoring was done by the same scorer as previously described (Griswold *et al.*, 1988).

Results

SB 220025 selectively inhibits p38 MAP kinase. The pyrimidyl imidazole compounds as exemplified by SB 203580 have been previously demonstrated to specifically inhibit p38 (Cuenda *et al.*, 1995; Lee *et al.*, 1994; Young *et al.*, 1997). SB 220025, a novel member of this structural class (fig. 1), was tested in a number of kinase assays to assess its use as a p38 inhibitor (table 1). This compound inhibited p38 phosphorylation of an EGFR peptide substrate with an IC$_{50}$ value of 60 nM, which is 10-fold more potent than SB 203580 (IC$_{50}$ = 0.6 μM; Cuenda *et al.*, 1995). In selectivity assays, p38 inhibition by SB 220025 was >1000-fold selective over Erk (p42/p44 MAP kinase), 500-fold selective over PKA, >1000-fold selective over EGFR and 50-fold selective over PKC.
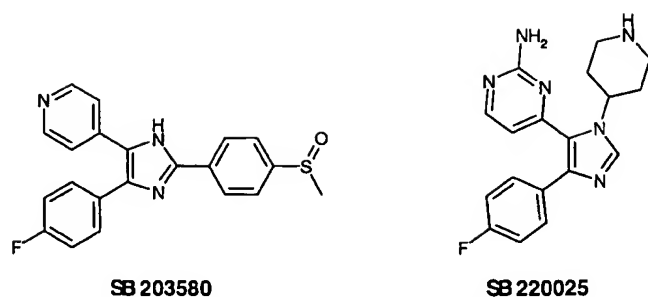
Fig. 1. Structures of SB 203580 and SB 220025.

SB 203580   **SB 220025**

TABLE 1

IC_{50} values for the effect of SB 220025 on the activities of protein kinases

	p38	Erk	PKA	PKC	EGFR	p56-Lck
SB 220025	0.060	>150	30.4	2.89	>100	3.5

Kinase assays were performed as described in the text. IC_{50} values are in μM and were calculated from percent inhibition data obtained using serial dilutions of the compound.

SB 220025 inhibits inflammatory cytokine production *in vivo.* To examine the *in vivo* efficacy of SB 220025, an acute model of LPS-induced TNF-α expression was used. SB 220025 at a range of doses from 3 to 50 mg/kg was given to mice orally 30 min before challenge with LPS. Serum TNF-α was measured by ELISA after 2 hr. This compound dose-dependently inhibited TNF-α production with an ED_{50} value of 7.5 mg/kg (fig. 2). Greater than 80% inhibition was obtained at 50 mg/kg. Thus, SB 220025 is an orally available, potent inhibitor of TNF-α synthesis

SB 220025 inhibits angiogenesis in the murine air pouch granuloma model. The inflammatory cytokines TNF-α and IL-1β are potent inducers of angiogenesis. To test whether they are involved in the promotion of angiogenesis in chronic inflammation, we used the cytokine-suppressive p38/CSBP MAP kinase inhibitor SB 220025 in a model of inflammatory angiogenesis. The murine air pouch granuloma has been characterized as a chronic inflammatory progression with a profound angiogenic component (Colville-Nash *et al.*, 1995). It provides a model in which modulation of angiogenesis in an inflammatory bed can be quantified. Granulomas were formed in a 3-ml dorsal subcutaneous air
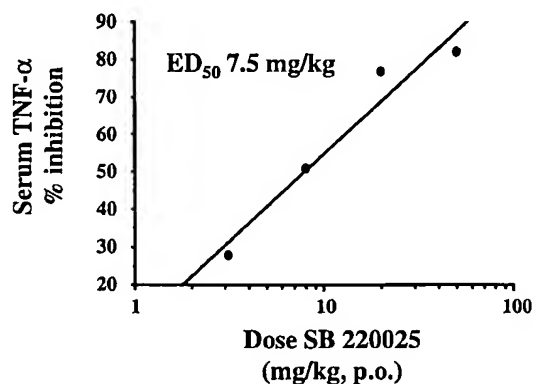
pouch by injection of 0.5 ml of Freund's complete adjuvant and croton oil. Within 3 days, a cohesive granulomatous tissue encased the adjuvant mixture. The granulomas were evaluated by weight, histology and vascular index (mg of carmine dye/g of dry tissue), which was used to assess the extent of angiogenesis.

Using a range of doses, we analyzed the effect of SB 220025 on granuloma size and vascular index on day 6. This time point was chosen because it allows sufficient time for angiogenesis and the development of chronic inflammatory character but occurs before the onset of fibrotic features (Colville-Nash *et al.*, 1995; Jackson *et al.*, 1997). The compound caused a dose-dependent reduction in angiogenesis as measured by the vascular index of the granuloma (fig. 3). The maximum effect was a 44% reduction at 50 mg/kg. This is similar to the maximum effect we obtained with a positive control, the angiostatic steroid medroxyprogesterone (fig. 3), which was chosen for its well-documented antiangiogenic activity (Gross *et al.*, 1981), lack of anti-inflammatory activity and consistent pharmacology in our experience with this model. Neither SB 220025 nor the angiostatic steroid had an effect on granuloma size (dry weight).

Effect of SB 220025 on the time course of angiogenesis. We evaluated the effect of the p38/CSBP inhibitor at several time points to determine whether its effects would be different at the various stages of inflammatory and angio-

Fig. 2. Effect of SB 220025 on LPS induction of TNF-α *in vivo.* Mice were pretreated with the indicated doses of SB 220025 and challenged with an intraperitoneal injection of LPS. TNF-α levels in the serum were measured 2 hr after the challenge by ELISA. The vehicle control TNF-α level was 4.35 ± 0.4 ng/mouse (n = 8). ED_{50} value was determined by regression analysis.

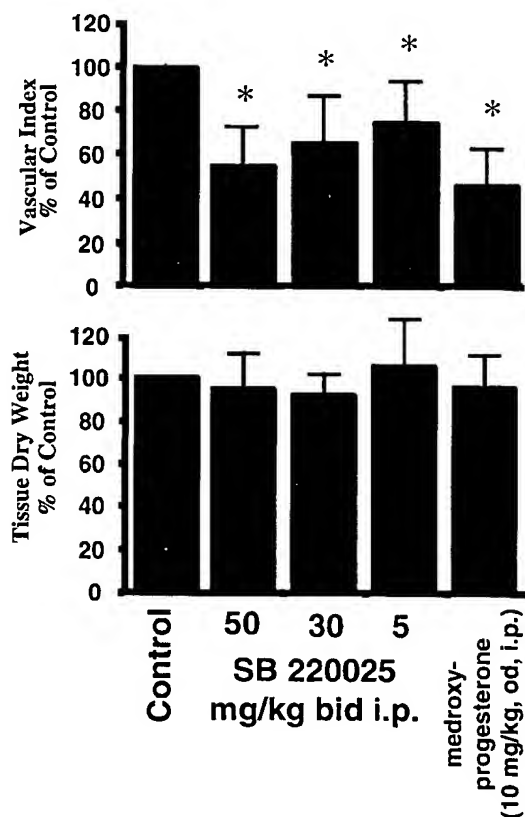**Fig. 3.** Tissue dry weight and vascular index (mg of carmine dye/g of dry tissue) of day 6 granulomas after treatment with SB 220025 or medroxyprogesterone. Dosing began when granulomas were induced. Data are expressed as a percentage of vehicle-treated control animals ± S.D. Control vascular index was 5.4, and control dry weight was 0.28 g (n = 5). *Significant from control at P < .05, calculated by Duncan's multiple-range test.

genic progression. SB 220025 was given orally, at an intermediate dose of 30 mg/kg twice a day starting on day 0, and granulomas were evaluated on days 3, 5, 7 and 14. Granuloma size remained fairly constant and was unaffected by the SB 220025 (fig. 4). The vascular index of the control group rose gradually from day 3 to 14, whereas the vascular index of the treated group remained constant. At day 3, the compound did not cause a significant reduction in vascular index compared with control; however, at days 5, 7 and 14, the vascular index was lowered significantly by SB 220025. Thus, the p38/CSBP MAP kinase inhibitor did not affect the initial burst of angiogenesis but did prevent the increase in angiogenesis that occurs after day 3.

Inflammatory cytokines such as IL-1β and TNF-α have been implicated in the pathogenesis of angiogenesis in chronic inflammation, and p38 inhibitors, such as SB 220025, have been demonstrated to inhibit the synthesis of these cytokines. We measured the levels of these cytokines over the course to granuloma development to determine whether the modulation of their expression by SB 220025 correlated with inhibition of angiogenesis. Cytokine levels were measured by ELISA using homogenates of granuloma tissue. TNF-α levels rose sharply, peaking at day 7 and dropping back down to moderate levels by day 14 (fig. 4). SB 220025 greatly reduced TNF-α levels at day 7. IL-β levels were also high in control granulomas, peaking at day 7, and as with TNF-α, the p38/CSBP MAP kinase inhibitor effectively blocked the increased IL-1β expression. Thus, the ability of SB 220025 to block the sharp rise in TNF-α and IL-1β between days 5 and 7 correlated well with the ability of the compound to prevent the increase in vascular index that occurs over the same time points.

Microscopic analysis angiogenesis in the granuloma. Angiogenesis in the granuloma was microscopically evaluated using cedarwood oil clearing. Figure 5 shows the vasculature of day 6 granulomas from both untreated and SB

220025-treated mice. The profound angiogenesis in the granuloma is demonstrated by the extensive vascular network in the control tissue. There was a striking reduction in the vasculature of the treated tissue. The fine capillaries seen in the control tissue were completely absent in the treated tissue, and only a few larger vessels remained visible.

Effect of SB 220025 on chronic inflammatory disease. The anti-inflammatory and antiangiogenic activities of SB 220025 suggest that it would provide an effective treatment in chronic inflammatory diseases such as rheumatoid arthritis, which has both inflammatory cytokine and angiogenic components. Thus, we tested SB 220025 in a chronic inflammatory disease model, murine collagen-induced arthritis. Mice were primed with bovine collagen, and 3 weeks later, the animals were given intraperitoneal injections of soluble collagen and monitored for the appearance of arthritis. Dosing began after arthritis was evident, usually between days 7 and 14 after collagen boost. The first day of dosing was designated day 0. Animals treated with SB 220025 (50 mg/kg p.o. b.i.d.) had no increase in severity of arthritis over 10 days, whereas the severity of arthritis in the control mice was increased at days 7 and 10 (fig. 6). Thus, the p38/CSBP MAP kinase inhibitor effectively blocked the progression of arthritis.

Discussion

Proinflammatory cytokines such as IL-1β and TNF-α have been shown to play a central role in many inflammatory processes (Dinarello, 1991). This study demonstrates the importance of IL-1β and TNF-α in chronic inflammatory angiogenesis and arthritis. Angiogenesis is a normal physiological response in wound healing, but in diseases such as rheumatoid arthritis and psoriasis, it can take on a pathological role. The association between angiogenesis and chronic inflammation has led to the hypothesis that angiogenesis is induced by inflammatory events. Indeed, it has been shown that IL-1β or TNF-α can induce angiogenesis in the normally avascular cornea (BenEzra et al., 1990; BenEzra and Maftzir, 1996; Fajardo et al., 1992). We evaluated the role of these cytokines in inflammatory angiogenesis in vivo by using a murine air pouch granuloma model. Both IL-1β and TNF-α levels in the granuloma tissue increased sharply over the first 7 days of granuloma formation, the same time period in which angiogenesis was very active.

We modulated the activity of IL-1β and TNF-α using the p38/CSBP inhibitor SB 220025, which inhibits their synthesis. This compound is more potent than the previously reported p38 inhibitor SB 203580. We observed an ED$_{50}$ value of 7.5 mg/kg for LPS-induced serum TNF-α production, which is twice as potent a value as that reported for SB 203580 (Badger et al., 1996). SB 220025 caused a significant dose-dependent decrease in the vascular density of the granuloma, and this correlated with decreases in IL-1β and TNF-α levels. The hypothesis is that decreasing IL1 and TNF-α levels resulted in inhibition of angiogenesis in an inflammatory tissue bed.

When we analyzed a time course of granuloma development, we observed that the control group granuloma size, as measured by dry weight, increased dramatically from day 0 to 3 and then was steady from day 3 to 14. In contrast, the control group vascular index increased steadily from day 3 to
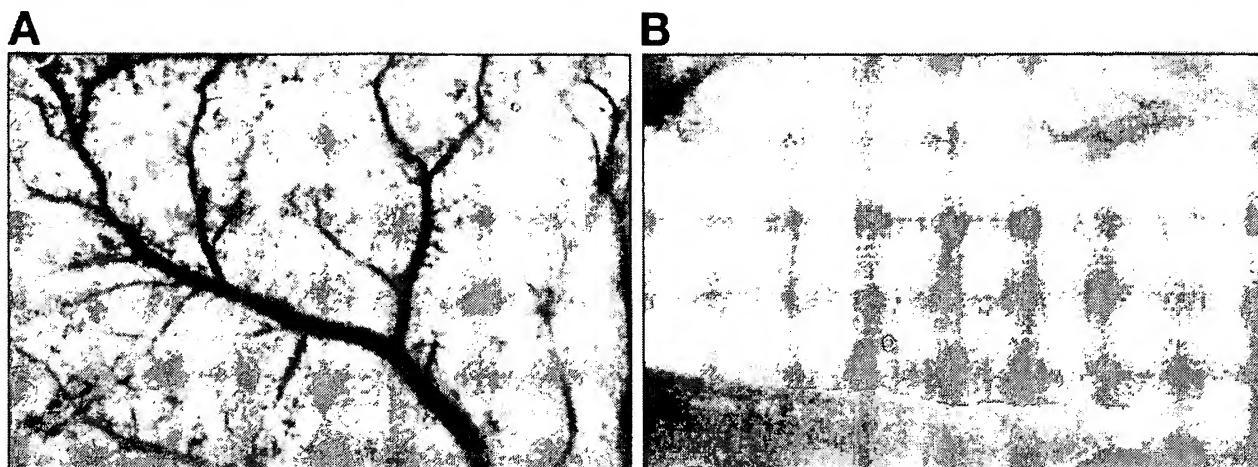
A

B

Fig. 5. Cedarwood oil histology of air pouch granuloma. Carmine dye vascular casts were made in day-6 granulomas from mice treated with SB 220025 (30 mg/kg b.i.d. p.o.) (A) or vehicle only (B). Granulomas were fixed in ethanol, cleared in cedarwood oil as described in the text, and photographed with transmitted light under 12× magnification.
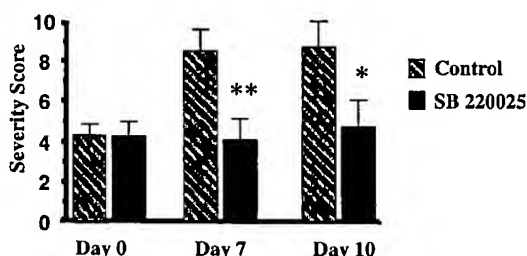

Fig. 6. Collagen-induced arthritis and measurement of severity are described in the text. Dosing with SB 220025 (50 mg/kg b.i.d. p.o.) or vehicle began only when the severity score reached ≈4, and this was designated day 0. Data are shown as mean ± S.D. (n = 7). *Significant from control at $P < .05$. **Significant from control at $P < .01$ calculated by Student's t test.

14. This time course is somewhat different from that reported by Colville-Nash *et al.* (1995), who observed a decrease in granuloma size after day 7, and biphasic vascular index, with a sharp peak at day 5 and a smaller peak at day 14. In other studies with this model, we observed a similar biphasic vascular index (data not shown); however, the dramatic maximum and minimum at days 5 and 7 appear to be very transient and have not been consistently reproducible. Thus, it is important to always run a control group for each time point in each drug study. Similar to Colville-Nash *et al.* (1995), we observed a regression in granuloma size, if followed beyond day 14.

In the present study, SB 220025 had no effect on granuloma size and did not inhibit angiogenesis at the 3-day time point, but it blocked the subsequent increases in vascularity most noticeably over days 5 and 7. This antiangiogenic activity correlates well with the highest levels of IL-1β and TNF-α in the granuloma. These time points also represent a switch from an acute to a chronic inflammatory phenotype (Jackson *et al.*, 1997), suggesting that the p38/CSBP MAP kinase inhibitor is most effective at inhibiting angiogenesis associated with chronic inflammation. The initial angiogenesis occurring over the first 3 days is likely to be induced by something other than IL-1β or TNF-α because their levels have not yet risen. One possibility is VEGF, which has been shown to be at its highest levels early in granuloma development (Appleton *et al.*, 1996). Moreover, at day 14 in the

control group, angiogenesis was still increasing in this study, even though TNF-α and IL-1β levels had dropped substantially. This time point represents another phenotypic change in the granuloma, when the chronic inflammatory phenotype gives way to a fibrotic phenotype (Jackson *et al.*, 1997). Thus, angiogenesis may be driven by another factor or factors, such as FGF, at this stage.

Granuloma size was not decreased by inhibition of angiogenesis with SB 220025. This was not surprising because granuloma growth is not angiogenesis dependent in this air pouch model (Colville-Nash *et al.*, 1995; Jackson *et al.*, 1997). The model provides an *in vivo* system for the study of hyperangiogenesis in an inflammatory tissue but is not a model of inflammatory disease. To test the effect of SB 220025 in a model of rheumatoid arthritis, an angiogenesis-dependent chronic inflammmatory disease, we used murine collagen-induced arthritis. Using a therapeutic dosing regimen, in which dosing did not begin until there was evidence of arthritic joint disease, SB 220025 was able to prevent further increases in the severity of arthritis. Thus, an inhibitor of IL-1β/TNF-α synthesis and angiogenesis was a very effective treatment for arthritis. This agrees with other studies that demonstrated that TNF-α antibodies (Piguet *et al.*, 1992) were an effective treatment for collagen arthritis and that the angiogenesis inhibitor AGM-1470 was able to reduce the severity of collagen-induced arthritis in rats (Peacock *et al.*, 1992). Interestingly, in a study of other anti-inflammatory drugs (Griswold *et al.*, 1988), the nonsteroidal anti-inflammatory drug ibuprofen was not particularly effective in this model, further suggesting that the anticytokine and antiangiogenic properties of SB 220025 are key to its antiarthritic activity.

Although inhibition of IL-1β and TNF-α synthesis is strongly implicated to be responsible for the antiangiogenic and antiarthritic activities of SB 220025, it is possible that inhibition of the synthesis of other cytokines also may be involved. Other factors, such as the inducible cyclooxygenase, IL-6, IL-8 and GM-CSF, also are regulated by p38/CSBP MAP kinase (Beyaert *et al.*, 1996; Lee *et al.*, 1988, 1989, 1993; Pouliot *et al.*, 1997) and thus may be affected by SB 220025. However, IL-1β and TNF-α are reported to have more potent

angiogenic activities than eicosanoids and these other cytokines, and the most effective antiangiogenic activity of SB 220025 on days 3 and 5 of granuloma development correlated well with inhibition of IL-1β and TNF-α synthesis. It is important to note that p38 inhibitors such as SB 203580 and SB 220025 also affect the signaling pathways of these cytokines and thus may work *via* inhibition of both cytokine synthesis and action (Badger *et al.*, 1996; Cuenda *et al.*, 1995). In addition, although it is a very selective inhibitor of p38 MAP kinase and we are unaware of any other activities that could account for its pharmacology, it is possible that SB 220025 may also inhibit an as-yet-unidentified kinase. Therefore, *in vivo* data should be interpreted with normal caution.

The association between inflammation and angiogenesis has long been observed, but until recently there has been little evidence to clearly demonstrate the link. This study shows that angiogenesis is dependent on inflammatory cytokines in a chronic inflammatory model. It is not clear whether inflammatory cytokines are involved in other angiogenesis dependent processes, such as tumor growth, and this remains to be tested. From our studies and others (Badger *et al.*, 1996), it is apparent that p38/CSBP MAP kinase inhibition should provide an effective treatment for chronic proliferative inflammatory diseases.

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A SHORT-TERM STUDY OF CHIMERIC MONOCLONAL ANTIBODY cA2 TO TUMOR NECROSIS FACTOR α FOR CROHN'S DISEASE

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ABSTRACT

Background Studies in animals and an open-label trial have suggested a role for antibodies to tumor necrosis factor α, specifically chimeric monoclonal antibody cA2, in the treatment of Crohn's disease.

Methods We conducted a 12-week multicenter, double-blind, placebo-controlled trial of cA2 in 108 patients with moderate-to-severe Crohn's disease that was resistant to treatment. All had scores on the Crohn's Disease Activity Index between 220 and 400 (scores can range from 0 to about 600, with higher scores indicating more severe illness). Patients were randomly assigned to receive a single two-hour intravenous infusion of either placebo or cA2 in a dose of 5 mg per kilogram of body weight, 10 mg per kilogram, or 20 mg per kilogram. Clinical response, the primary end point, was defined as a reduction of 70 or more points in the score on the Crohn's Disease Activity Index at four weeks that was not accompanied by a change in any concomitant medications.

Results At four weeks, 81 percent of the patients given 5 mg of cA2 per kilogram (22 of 27 patients), 50 percent of those given 10 mg of cA2 per kilogram (14 of 28), and 64 percent of those given 20 mg of cA2 per kilogram (18 of 28) had had a clinical response, as compared with 17 percent of patients in the placebo group (4 of 24) ($P < 0.001$ for the comparison of the cA2 group as a whole with placebo). Thirty-three percent of the patients given cA2 went into remission (defined as a score below 150 on the Crohn's Disease Activity Index), as compared with 4 percent of the patients given placebo ($P = 0.005$). At 12 weeks, 41 percent of the cA2-treated patients (34 of 83) had had a clinical response, as compared with 12 percent of the patients in the placebo group (3 of 25) ($P = 0.008$). The rates of adverse effects were similar in the groups.

Conclusions A single infusion of cA2 was an effective short-term treatment in many patients with moderate-to-severe, treatment-resistant Crohn's disease. (N Engl J Med 1997;337:1029-35.)

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CROHN'S disease is a chronic inflammatory disorder characterized by patchy granulomatous inflammation of any part of the gastrointestinal tract.[1] Patients have a spectrum of clinical features, with great variation in the course of the disease. Mesalamine is considered first-line therapy. The majority of patients have relapses requiring glucocorticoid treatment.[2] Immunomodulatory agents, including azathioprine or mercaptopurine,[3] methotrexate,[4,5] and cyclosporine,[6-9] may be used to treat severe, persistent disease that is refractory to treatment with corticosteroids, or symptoms that recur on tapering of the dose of corticosteroids.

In animal models, antibodies to tumor necrosis factor α (anti–TNF-α) prevent or reduce inflammation,[10-14] suggesting that therapy with such antibodies may be useful for disorders in which chronic inflammation may be due to an increase in cytokines produced by the T helper 1 subclass of T cells. In vitro studies have shown that the production of TNF-α is increased in the mucosa of patients with Crohn's disease[15,16] and that the mucosal inflammatory process reflects a shift in the balance of cytokine production by T cells toward the T helper 1 subclass.[17,18] Similar findings were reported in the synovia of patients with rheumatoid arthritis,[19] and anti–TNF-α reduces clinical signs and symptoms of this disease.[20,21] The role of TNF-α in the pathogenesis of Crohn's disease and the successful use of anti–TNF-α in the treatment of rheumatoid arthritis stimulated an open-label trial of chimeric monoclonal antibody cA2 (infliximab, Centocor, Malvern, Pa.) for Crohn's disease. In that preliminary trial, clinical remission occurred after one infusion of cA2 in eight of nine patients with Crohn's disease.[22] We report the results of a multicenter randomized, placebo-controlled, double-blind trial of cA2 for the treatment of active Crohn's disease.

METHODS

Patients

To be eligible for the study, patients had to have had Crohn's disease for six months,[1] with scores on the Crohn's Disease Activity Index[23] between 220 and 400. The Crohn's Disease Activity Index incorporates eight variables related to the disease: the number of liquid or very soft stools, the severity of abdominal pain or cramping, general well-being, the presence of extraintestinal man-

ifestations, abdominal mass, use of antidiarrheal drugs, hematocrit, and body weight. These items yield a composite score ranging from 0 to approximately 600. Higher scores indicate greater disease activity. Scores below 150 indicate remission, whereas scores above 450 indicate severe illness. Patients were eligible for the study if they had been receiving any of the following: mesalamine for eight or more weeks, with the dose remaining stable during the four weeks before screening; a maximum of 40 mg of corticosteroids per day for eight or more weeks, with the dose remaining stable during the two weeks before screening; and mercaptopurine or azathioprine for six or more months, with the dose remaining stable during the eight weeks before screening. Patients were excluded from the study if they had received treatment with cyclosporine, methotrexate, or experimental agents within three months before screening. Patients were also excluded if they met any of the following criteria: symptomatic stenosis or ileal strictures; proctocolectomy or total colectomy; stoma; a history of allergy to murine proteins; prior treatment with murine, chimeric, or humanized monoclonal antibodies; or treatment with parenteral corticosteroids or corticotropin within four weeks before screening.

Patients were enrolled at 18 centers in North America and Europe. The protocol was approved by the institutional review boards and ethics committees at all sites, and all patients gave written informed consent before enrolling in the trial. The study began on June 21, 1995, and concluded on March 12, 1996. A total of 203 patients were screened for the study, 95 of whom were excluded. The most common reasons for exclusion were a requirement for contraindicated medications, refusal to give informed consent, or disease activity that did not meet the study criteria.

Protocol

Subjects were screened one week before the administration of cA2 to establish base-line scores on the Crohn's Disease Activity Index and the Inflammatory Bowel Disease Questionnaire,[24] and base-line C-reactive protein concentrations. The Inflammatory Bowel Disease Questionnaire, a 32-item questionnaire, evaluates quality of life with respect to bowel function (e.g., loose stools and abdominal pain), systemic symptoms (fatigue and altered sleep pattern), social function (work attendance and the need to cancel social events), and emotional status (angry, depressed, or irritable). The score ranges from 32 to 224, with higher scores indicating a better quality of life. Patients in remission usually score between 170 and 190.[24]

Patients were randomly assigned to receive a single dose of either placebo or 5 mg of cA2 per kilogram of body weight, 10 mg of cA2 per kilogram, or 20 mg of cA2 per kilogram in an intravenous infusion, administered over a two-hour period. The cA2 monoclonal antibody is a chimeric mouse–human IgG1 that binds to both soluble[25] and transmembrane[26] human TNF-α with high affinity and specificity. It neutralizes the functional activity of TNF in a variety of bioassays by blocking the binding of the factor to the p55 and p75 receptors.[27] The placebo preparation contained 0.1 percent human serum albumin instead of cA2 and was identical in appearance to the cA2 solution. Patients were enrolled from June 21, 1995, to October 31, 1995. Randomization was performed centrally by an independent organization (PPD Pharmaco, Austin, Tex.). The cA2 and placebo solutions were prepared by a pharmacist at each site who was aware of the treatment assignments. The investigators, all other study personnel, and the patients were blinded to the treatment assignments.

The primary end point was defined before the initiation of the trial as a reduction of 70 points or more in the score on the Crohn's Disease Activity Index at the four-week evaluation that was not accompanied by a change in any concomitant medications. Patients who did not have a clinical response at that time were enrolled in a parallel, open-label study and received a single infusion of 10 mg of cA2 per kilogram and were followed for 12 additional weeks. Patients who were receiving mesalamine, corti-

costeroids, azathioprine, or mercaptopurine before the study continued to receive a stable dose during the trial period. The dose of corticosteroids could be tapered beginning eight weeks after the initiation of the study. Treatment with these drugs or with methotrexate or cyclosporine could not be initiated during the trial. After all patients had completed 12 weeks of the trial and the data were finalized, the treatment assignments were revealed.

Immunologic Investigations

Serum samples were obtained at base line and at 12 weeks for the evaluation of antinuclear antibodies and human anti-cA2. Antinuclear antibodies were detected by immunofluorescence on Hep-2 cells. Serum samples positive by immunofluorescence for antinuclear antibodies were tested for antibodies to double-stranded DNA by an enzyme-linked immunosorbent assay (North American centers) or by Crithidia immunofluorescence (European centers). Human anti-cA2 was measured by a double-antigen enzyme-linked immunosorbent assay.

Statistical Analysis

An adaptive stratified design was used to assign patients to a treatment group, with investigational site and corticosteroid use as the strata. We calculated that approximately 25 patients were needed in each treatment group to detect a difference in the number of patients who responded with 80 percent power ($\alpha = 0.05$), assuming a response rate of 30 percent in the placebo group, 80 percent in the cA2 group with the greatest response, and 55 percent in the remaining cA2 groups. The original study protocol did not specify the use of intention-to-treat analysis. Two patients were assigned to a treatment but did not receive it: one declined to participate and one did not meet eligibility criteria. No further data were collected on these two patients, and they are not included in the analysis. Otherwise, all patients were analyzed according to the treatment to which they were assigned. When we assessed the response or remission rates in all evaluation periods after the initial blinded infusion, patients who received an open-label infusion or those with a change in concomitantly administered medications were considered to have had no response.

Categorical variables (clinical response and remission) were compared with use of the Mantel–Haenszel chi-square test for general association stratified according to investigational site.[28] Analyses comparing each of the cA2 treatment groups with placebo were performed only when the treatment effect was considered significant (P<0.05). The changes from base line in continuous variables (Crohn's Disease Activity Index score, Inflammatory Bowel Disease Questionnaire score, and C-reactive protein concentration) were compared with use of analysis of variance, with the van der Waerden normal scores blocked according to center.[29] If the treatment effect was significant, the cA2 treatment groups were compared with the placebo group with linear contrasts. All P values are two-sided.

RESULTS

Base-Line Characteristics of the Study Patients

A total of 108 patients were studied, with 25 to 28 patients in each group. There were no significant differences in age, weight, race (all patients were white), sex, duration of disease, scores on the Crohn's Disease Activity Index and Inflammatory Bowel Disease Questionnaire, or C-reactive protein concentrations at base line among the groups, although patients in the placebo group had a lower mean concentration of C-reactive protein (Table 1). Significantly more patients had ileal disease alone in the placebo group than in the other three groups (P = 0.02), but there were no significant differences

TABLE 1. BASE-LINE CHARACTERISTICS OF THE 108 PATIENTS.*

CHARACTERISTIC	PLACEBO (N=25)	5 mg OF cA2/kg (N=27)	10 mg OF cA2/kg (N=28)	20 mg OF cA2/kg (N=28)
Duration of disease — yr	10.4±7.7	12.5±10.3	11.5±9.6	13.5±8.8
Involved intestinal area — no. of patients (%)				
Ileum only	8 (32)†	3 (11)	4 (14)	2 (7)
Ileum and colon	10 (40)	15 (56)	14 (50)	19 (68)
Colon only	7 (28)	9 (33)	10 (36)	7 (25)
Previous segmental resection — no. of patients (%)	13 (52)	12 (44)	14 (50)	14 (50)
Age — yr	38.5±11.0	37.0±11.8	39.3±10.6	36.0±9.7
Male sex — no. (%)	15 (60)	14 (52)	13 (46)	13 (46)
Weight — kg	71.4±14.4	68.1±17.7	74.2±19.5	68.4±16.0
Height — cm	172±11	169±8	171±10	171±9
Medications — no. of patients				
Prednisone equivalent				
<20 mg/day orally	10 (40)	8 (30)	8 (29)	10 (36)
≥20 mg/day orally	6 (24)	7 (26)	8 (29)	7 (25)
Mercaptopurine	4 (16)	4 (15)	4 (14)	4 (14)
Azathioprine	7 (28)	5 (19)	4 (14)	8 (29)
Oral aminosalicylates	17 (68)	16 (59)	18 (64)	13 (46)
Score on Crohn's Disease Activity Index	288±54	312±56	318±59	307±50
Score on Inflammatory Bowel Disease Questionnaire	128±29	122±29	116±23	118±28
C-reactive protein — mg/liter	12.8±13.9	22.1±23.6	23.2±34.2	22.4±23.9

*Plus–minus values are means ±SD. Higher scores on the Crohn's Disease Activity Index indicate greater disease activity, and higher scores on the Inflammatory Bowel Disease Questionnaire indicate better quality of life.

†P = 0.02 for the comparison with the other three groups.

in the number who had undergone previous segmental resections among the groups. Similar numbers of patients in each group had been treated with oral corticosteroids, mercaptopurine, azathioprine, and oral mesalamine at base line. All treatment groups had a mean score on the Crohn's Disease Activity Index of approximately 300, despite concurrent treatment with drugs other than cA2; thus, the patients had moderate-to-severe, treatment-resistant Crohn's disease.

Clinical Response and Remission

Week 2

Figure 1A demonstrates that clinical response was achieved early: 61 percent of cA2-treated patients had a clinical response by week 2, as compared with 17 percent of patients in the placebo group (P< 0.001). At two weeks, 27 percent of cA2-treated patients were in clinical remission (defined as a score of less than 150 on the Crohn's Disease Activity Index), as compared to 4 percent of the patients in the placebo group (P=0.06) (Fig. 1B).

Week 4

Four weeks after the infusion, the primary end point of a reduction of 70 or more points in the score on the Crohn's Disease Activity Index was reached in 81 percent of those given 5 mg of cA2 per kilogram (22 of 27 patients), 50 percent of those given 10 mg of cA2 per kilogram (14 of 28 patients), and 64 percent of those given 20 mg of cA2 per kilogram (18 of 28 patients), as compared with 17 percent of those given placebo (4 of 24 patients) (Fig. 1A). The overall response of the cA2 groups was 65 percent (P<0.001 for the comparison with placebo). No dose–response relation was seen during this period. At four weeks, 33 percent of the cA2-treated group were in remission, as compared with 4 percent of the placebo group (P = 0.005). Thus approximately half of the patients who had a clinical response at either two or four weeks also entered remission. Consistent treatment effects were observed when the analyses for both response and remission at week 4 were stratified according to the location of disease or concurrent drug regimens (data not shown).

Changes in Clinical and Inflammatory Measures during the First Four Weeks

The mean change in the scores on the Crohn's Disease Activity Index in the cA2-treated group as a whole was significant at weeks 2 and 4 of the trial, as compared with the changes in scores in the placebo group (P<0.001) (Table 2). The mean decrease in the score on the Crohn's Disease Activity Index
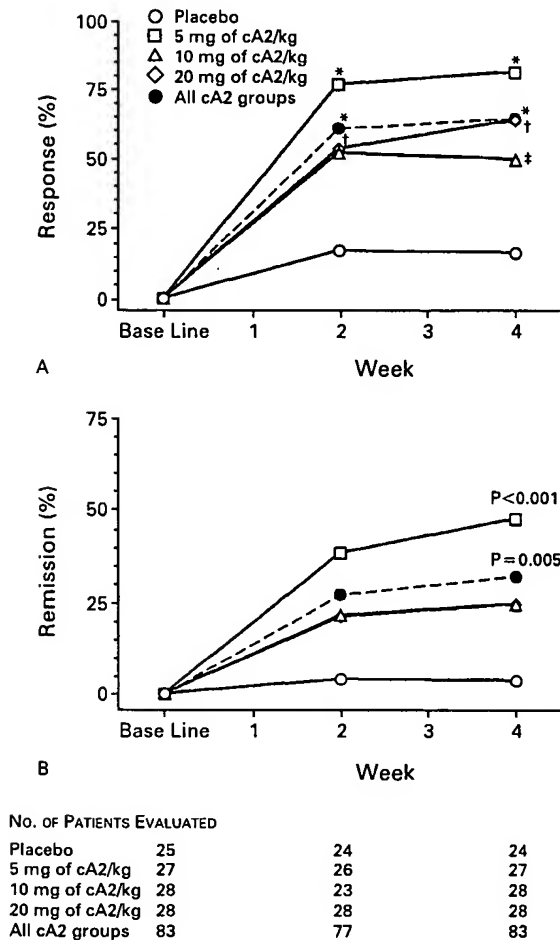
A

B

No. of Patients Evaluated

Placebo	25	24	24
5 mg of cA2/kg	27	26	27
10 mg of cA2/kg	28	23	28
20 mg of cA2/kg	28	28	28
All cA2 groups	83	77	83

Figure 1. Rates of Clinical Response and Remission after a Single Infusion of cA2 or Placebo.

Clinical remission was defined as a score of less than 150 on the Crohn's Disease Activity Index and a score of 170 to 190 on the Inflammatory Bowel Disease Questionnaire. The asterisks (P<0.001), daggers (P<0.01), and double dagger (P<0.05) indicate a significant difference from placebo.

in the cA2 group as a whole was 110 at four weeks, as compared with 13 in the placebo group. Most of this decrease had occurred by week 2, with a mean decrease of 103 in the cA2 group and 16 in the placebo group.

The Inflammatory Bowel Disease Questionnaire was given at base line and four weeks. There was a mean increase of 46, 30, and 32 in the groups treated with cA2 at 5, 10, and 20 mg of cA2 per kilogram, respectively, yielding a mean increase of 36 in the cA2 group as a whole, as compared with a mean increase of 5 in the placebo group (P=0.001) (Table 2).

Concentrations of C-reactive protein were measured at base line and weeks 2 and 4. At four weeks,

the mean decrease in C-reactive protein was 16.3, 11.1, and 15.0 mg per liter in the groups treated with 5, 10, and 20 mg of cA2 per kilogram, respectively, yielding a mean decrease of 14.3 mg per liter in the group as a whole, as compared with a mean increase of 2.0 mg per liter in the placebo group (P<0.001). The maximal reduction in C-reactive protein occurred within the first two weeks. At two weeks, the mean decrease in C-reactive protein in the cA2 groups as a whole was 16.0 mg per liter, as compared with a mean increase of 3.9 mg per liter in the placebo group (P<0.001).

Week 12

The differences in the rates of clinical response between the cA2-treated groups and the placebo group remained significant through the 12 weeks of follow-up: it was 48 percent in the group given 5 mg of cA2 per kilogram (13 of 27 patients), 29 percent in the group given 10 mg of cA2 per kilogram (8 of 28 patients), and 46 percent in the group given 20 mg of cA2 per kilogram (13 of 28 patients), for an overall rate of response of 41 percent (34 of 83 patients), as compared with a rate of 12 percent in the placebo group (3 of 25 patients) (P=0.008). The difference in the percentage of patients who were in remission was not significant at 12 weeks: 30 percent of the group given 5 mg of cA2 per kilogram (8 of 27 patients), 18 percent of the group given 10 mg of cA2 per kilogram (5 of 28 patients), and 25 percent of the group given 20 mg of cA2 per kilogram (7 of 28 patients), for an overall rate of remission achieved of 24 percent (20 of 83 patients), as compared with a rate of 8 percent in the placebo group (2 of 25 patients) (P=0.31).

Characterization of the Response to Treatment

The magnitude and duration of response were characterized through the 12-week follow-up period in the 54 patients who responded to a single infusion of cA2. The improvement in the scores on the Crohn's Disease Activity Index (clinical remission was defined as a score below 150) and Inflammatory Bowel Disease Questionnaire (remission was defined as a score between 170 and 190) in patients with a response was maintained. The mean (±SD) score on the Crohn's Disease Activity Index was 318±52 at base line, 144±67 at week 4, 151±86 at week 8, and 182±91 at week 12, and the mean score on the Inflammatory Bowel Disease Questionnaire was 121±26 at base line, 175±26 at week 4, 165±36 at week 8, and 162±35 at week 12. Concentrations of C-reactive protein (normal, <8 mg per liter) began to rise at 12 weeks, potentially indicating a relapse of disease (from 25.8±2.7 mg per liter at base line to 7.5±1.5 mg per liter at week 4, 11.0±2.1 mg per liter at week 8, and 14.1±2.2 mg per liter at week 12).

TABLE 2. MEASURES OF CLINICAL RESPONSE AND INFLAMMATION AT BASE LINE
AND WEEKS 2 AND 4.*

VARIABLE	PLACEBO (N=25)	5 mg OF cA2/kg (N=27)	10 mg OF cA2/kg (N=28)	20 mg OF cA2/kg (N=28)	ALL cA2 GROUPS (N=83)
Score on Crohn's Disease Activity Index					
Base line	288±54	312±56	318±59	307±50	312±55
2 weeks	272±75	182±79†	238±92‡	217±92†	212±90†
4 weeks	271±82	166±76†	226±115§	211±107†	201±103†
Score on Inflammatory Bowel Disease Questionnaire					
Base line	128±29	122±29	116±23	118±28	118±27
4 weeks	133±28	168±36†	146±41¶	149±35‖	154±38‡
C-reactive protein (mg/liter)					
Base line	12.8±13.9	22.1±23.6	23.2±34.2	22.4±23.9	22.6±27.4
2 weeks	16.4±18.9	4.2±3.0†	6.7±7.3**	8.7±13.8†	6.5±9.3†
4 weeks	14.8±18.6	5.7±9.3††	12.1±18.6§	6.9±11.6†	8.3±13.9†

*Plus–minus values are means ±SD. Higher scores on the Crohn's Disease Activity Index indicate greater disease activity, and higher scores on the Inflammatory Bowel Disease Questionnaire indicate better quality of life. All P values are for the change from base line in cA2-treated groups as compared with the placebo group. See the Methods section for a description of the statistical analyses used.

†P<0.001.	‡P=0.001.	§P=0.003.	¶P=0.02.
‖P=0.03.	**P=0.002.	††P=0.004.	

Effects of Open-Label cA2 in Patients with No Response Four Weeks after the Initial Infusion of cA2

Patients who did not have a clinical response after the first infusion were given a second infusion of open-label cA2 in a dose of 10 mg per kilogram and followed for an additional 12 weeks. Among 19 patients who initially received placebo, the response rate was 58 percent and the remission rate was 47 percent four weeks after the second infusion — rates that were similar to those in the initial, blinded study (Fig. 1). By contrast, among the 29 patients who had no response to the initial cA2 infusion, the rates of response and remission after the second infusion were 34 percent (P=0.14 for the comparison with the patients who received placebo initially) and 17 percent (P=0.05), respectively, confirming that this group was less responsive to cA2.

Adverse Effects

Adverse effects were recorded at the time of infusion and 2, 4, 8, and 12 weeks after the infusion. In patients who received an open-label infusion 4 weeks after an infusion of placebo or cA2, adverse effects were monitored for an additional 12 weeks. As shown in Table 3, the percentages of patients with adverse effects were similar in the placebo and cA2 groups. Of the 29 patients who received two cA2 infusions, 2 had a reaction (chest pain, dyspnea, or nausea) that led to the discontinuation of the infusion. These reactions resolved spontaneously within minutes after the infusion was discontinued. In addition, complications requiring hospitalization developed in two patients (abdominal abscess in one

patient in the placebo group and salmonella colitis in one patient given an infusion of 20 mg of cA2 per kilogram). Both patients were treated successfully. A bowel obstruction occurred in one patient given cA2, and a flare of Crohn's disease occurred in another.

Immunologic Responses

Serum samples were screened for antibodies to double-stranded DNA at base line and at 12 weeks in 98 patients who received cA2 (in either a blinded

TABLE 3. ADVERSE EFFECTS.*

VARIABLE	PLACEBO	ONE DOSE OF cA2	TWO DOSES OF cA2
No. of patients evaluated	25	102	29
Average length of follow-up — wk	6.9	10.4	12.4
Adverse effect — no. of patients (%)			
Any adverse effect	15 (60)	76 (75)	23 (79)
Headache	5 (20)	19 (19)	3 (10)
Nausea	2 (8)	11 (11)	5 (17)
Upper respiratory tract infection	3 (12)	8 (8)	4 (14)
Fatigue	1 (4)	6 (6)	3 (10)
Myalgia	1 (4)	4 (4)	3 (10)
Rhinitis	1 (4)	3 (3)	3 (10)
Pain	0	4 (4)	3 (10)
Pruritus	1 (4)	1 (1)	4 (14)
Chest pain	1 (4)	2 (2)	3 (10)
Vomiting	0	2 (2)	3 (10)
Dyspnea	0	1 (1)	3 (10)

*Adverse effects that occurred in 10 percent or more of the patients in any of the groups are reported.

or an open-label infusion); all patients were negative for these antibodies at base line, and 3 were positive at 12 weeks. Serum samples from 101 patients treated with cA2 (in either a blinded or an open-label infusion) were also tested for human anti-cA2; 6 patients tested positive. In two thirds of the patients, however, cA2 was still detectable in serum samples taken at 12 weeks, and this may have interfered with the assay.

DISCUSSION

Many of our patients with moderate-to-severe Crohn's disease that was resistant to treatment had a rapid response to cA2. Other treatments for such patients have not had beneficial effects over the long term and may not be well tolerated.[30-33] Our short-term study suggests that anti–TNF-α therapy with cA2 may represent a new treatment option for patients with moderate-to-severe Crohn's disease. Further studies will be necessary to determine the long-term efficacy of a single infusion of cA2 as well as the efficacy and safety of repeated treatments.

The mechanisms of the chronic mucosal inflammatory processes manifesting as Crohn's disease are not clear. However, our results add strength to the suggestion that TNF-α may have a central role in the inflammatory process in at least two thirds of the patients with Crohn's disease.[22] Studies in rodent models have provided insight into the role of anti–TNF-α in the pathogenesis of Crohn's disease. Studies involving the transfer of CD45RB^{high} cells to mice with severe combined immunodeficiency have shown that these cells can induce a chronic, transmural inflammatory process in the colon.[34] Powrie et al. have demonstrated that this colitis can be reversed or ameliorated by the use of agents, including anti–TNF-α, capable of down-regulating the production of the T helper 1 subclass of T cells.[10] These cells produce proinflammatory cytokines including interferon-γ, interleukin-2, and potentially, TNF. A mechanistic role for TNF-α in intestinal inflammation has been suggested on the basis of these studies.

Other studies involving animal models have shown that cytokines produced by the T helper 1 subclass are expressed in the mucosa of people with Crohn's disease.[17,18] Anti–TNF-α may participate in the down-regulation of mucosal inflammation in this disease by inhibiting the T helper 1 population of active T cells. Extensive follow-up studies are needed to determine how the elimination of TNF affects mucosal inflammation after treatment with anti–TNF-α.

In this study, a clinical response or remission occurred in 65 percent of patients with severe Crohn's disease after a single infusion of cA2. Furthermore, results in the open-label retreatment phase corroborated the finding that patients with no response to the first infusion of cA2 were less likely to have a response to a second infusion. Thus, this group of patients may differ from those that responded. The similarities among the patients, including age, duration of disease, types of concomitant treatment, and disease activity at base line, suggest that any differences are more likely to be detected subclinically. There was no apparent dose–response relation between a dose of 5 mg of cA2 per kilogram and a dose of 20 mg per kilogram with respect to either the magnitude or the duration of the clinical response. In a previous open-label trial of cA2 doses of 1 mg, 5 mg, 10 mg, and 20 mg per kilogram, the group receiving 1 mg of cA2 per kilogram had a more transient response than the groups given higher doses.[35] This transient response was similar to that in a small trial of a different anti–TNF-α antibody.[36] The results of these trials support the use of a dose of 5 mg of cA2 per kilogram in future trials.

In summary, we found that a single infusion of cA2 was an effective short-term treatment for patients with moderate-to-severe Crohn's disease that was resistant to treatment.

APPENDIX

The Crohn's Disease cA2 Study Group consists of the following centers and investigators (the number of patients enrolled at each center is given in parentheses): *Massachusetts General Hospital, Boston* (5 patients): D. Podolsky, B.E. Sands, M.T. Marcucci; *Cedars–Sinai Medical Center, Los Angeles* (20 patients): S.R. Targan, E.A. Vasiliauskas, B. Voigt, J. Gaiennie; *University of Chicago Hospitals and Clinics, Chicago* (11 patients): S.B. Hanauer; *University of Alabama School of Medicine, Birmingham* (2 patients): C.O. Elson, R.P. McCabe, Jr.; *Mount Sinai Medical Center, New York* (8 patients): L. Mayer, D.H. Present, C. Stamaty; *Washington University School of Medicine, St. Louis* (2 patients): W.F. Stenson, J.J. O'Brien; *Virginia Mason Medical Center, Seattle* (5 patients): R. Kozarek, M. Gelfand; *Hospital of the University of Pennsylvania, Philadelphia* (4 patients): D. Bachwich, G. Lichtenstein, L. Hurd; *McMaster University Medical Center, Hamilton, Ont., Canada* (2 patients): E.J. Irvine, S. Collins; *Lahey Clinic, Burlington, Mass.* (3 patients): A.S. Warner, L.J. Costa; *University of North Carolina, Chapel Hill* (5 patients): K.L. Isaacs; *University of Maryland Medical System, Baltimore* (5 patients): S. James, B. Greenwald, M.L. Mullen; *University of Kentucky, Lexington* (5 patients): G.W. Varilek, B. Vivian; *Academisch Ziekenhuis Leiden, Leiden, the Netherlands* (4 patients): R.A. van Hogezand, M.J. Wagtmans; *Institute for Clinical Immunology and Rheumatology, Erlangen, Germany* (1 patient): H. Schönekäs, J.R. Kalden, J.M.L. Bauer; *University of Amsterdam, Amsterdam* (9 patients): S.J.H. van Deventer, C.M.J. Kothe, O.J.B. de Smit; *Leeds General Infirmary, Leeds, United Kingdom* (4 patients): D.M. Chalmers, S. Chitturi, D. Todi; and *Academisch Ziekenhuis Gasthuisberg, Leuven, Belgium* (13 patients): P.J. Rutgeerts, G.R.A.M. D'Haens, A.F.M. Verstraeten.

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Treatment of ulcerative colitis in the cottontop tamarin using antibody to tumour necrosis factor alpha

PE Watkins, BF Warren, S Stephens, P Ward and R Foulkes

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Notes

Treatment of ulcerative colitis in the cottontop tamarin using antibody to tumour necrosis factor alpha

P E Watkins, B F Warren, S Stephens, P Ward, R Foulkes

Abstract

Background—The aetiology and pathophysiology of ulcerative colitis remains unclear; however, there is increasing recognition of the critical role of inflammatory cytokines in the pathogenesis of this disease. Among these, tumour necrosis factor α (TNFα) seems to play an important role.

Aim—To study the effects of an engineered human monoclonal antibody to TNFα (CDP571) in the treatment of idiopathic ulcerative colitis in the cottontop tamarin.

Methods—Six cottontop tamarins with confirmed ulcerative colitis received repeated doses of CDP571. Progression of disease was assessed by measuring both body weight and rectal biopsy pathology.

Results—All animals showed a rapid improvement in clinical condition and rectal biopsy pathology that was maintained following completion of the therapy.

Conclusion—These studies indicate the efficacy of selective antibody therapy to TNFα for the treatment of ulcerative colitis in a primate and suggest that similar therapy in humans could be of value.
(*Gut* 1997; 40: 628–633)

Keywords: ulcerative colitis, cottontop tamarin, antibody to tumour necrosis factor α

The term inflammatory bowel disease (IBD) encompasses two chronic disorders affecting all or part of the gastrointestinal tract. These are Crohn's disease and ulcerative colitis. For both diseases the causative agent is unclear. Proposed aetiologies include infectious agents,[1] [2] response to measles vaccination,[3] vascular disruptions,[4] and disorders of the immune system. Furthermore, there is increasing evidence of a genetic susceptibility to these diseases, which may act alone or perhaps in association with environmental factors.[5]

Ulcerative colitis and Crohn's disease both demonstrate characteristic clinicopathological features. A central feature is the local inflammatory response in the gut wall which is characterised by an influx of T-lymphocytes and other mononuclear cells, especially macrophages.[6] In recent years, numerous cytokines have been implicated as being crucial to the disease processes, including tumour necrosis factor α (TNFα), interleukin (IL) 6, IL-1β,[7] and platelet activating factor (PAF).[8] However,

the significance of individual cytokines has often been difficult to elucidate from clinical studies. At times, investigators have failed to show a consistent alteration in cytokine production or release in IBD. Furthermore, in patients receiving treatment, certain treatments – for example, 5-aminosalicylic acid or corticosteroids, or both, will cause a reduction in the concentrations of numerous cytokines in the colonic mucosa.[9] More recently, the development of specific antibodies to cytokines such as TNFα has provided the opportunity to assess the possible roles of individual cytokines in the disease state both in animal models and in humans.

TNFα is a cytokine released by activated mononuclear cells and T cells. It seems to have a clinically important role in septic shock[10] and in rheumatoid arthritis.[11] In the latter condition selective blockade of TNFα by monoclonal antibodies will ameliorate the disease process.[12] In addition, TNFα has been implicated in the pathogenesis of IBD. Raised concentrations of TNFα have been noted in the serum[13] and increased TNFα immunoreactivity has been shown in the lamina propria of patients with active Crohn's disease or ulcerative colitis.[14] Furthermore, it has been shown that there are significant increases in faecal TNFα concentrations in patients with active IBD.[15]

A large number of animal models of IBD have been described and many have been reviewed previously.[16] In most cases the models are solely of colonic inflammation, not of IBD. Inflammation is often induced by the local application of an irritant, such as acetic acid, to the colon, in some cases with prior sensitisation of the animal to the irritant material. Although these approaches can be used to study various aspects of colonic inflammation, the resulting pathology shows few, if any, similarities to that seen in human IBD. Moreover, the pathological changes invariably improve once the insult has been removed, unlike the situation in humans. None the less, in a rodent model of colonic inflammation a beneficial response was noted after administration of a selective monoclonal antibody directed against TNFα.[17]

The cottontop tamarin, a small, new world primate, is unique among animal models of IBD in that it develops a spontaneous form of colitis which shows many similarities to the condition of ulcerative colitis in humans. Animals present clinically with chronic diarrhoea and weight loss and may die from the

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condition if untreated. Both the pathology and the response to therapy with 5-aminosalicylic acid compounds, in some cases combined with corticosteroids, show close similarities to the condition in humans.[18] [19] A unique feature of the disease in the cottontop tamarin is the development of secondary complications, namely colonic adenocarcinoma[20] and sclerosing cholangitis,[21] which are also seen in human ulcerative colitis. Also, we have recently demonstated a significant increase in faecal TNFα concentrations in cottontop tamarins with active colitis,[22] illustrating a further similarity to the condition in humans.

The cottontop tamarin has been used before to evaluate potential therapeutic regimens for treating ulcerative colitis. These trials have included use of murine monoclonal antibodies against the adhesion molecules E-selectin and α4 integrin.[23]

In this study the effect of an engineered monoclonal antibody against human TNFα (CDP571) was assessed in spontaneous ulcerative colitis in the cottontop tamarin. The study was divided into two sections: first, the pharmacokinetics of CDP571 were evaluated in two normal animals; and, second, a trial using CDP571 for treatment of ulcerative colitis in the cottontop tamarin was undertaken. This study was planned and executed in a similar way to an "open" clinical trial. This was because of the comparative scarcity of the amimals (which are listed as endangered under the Conference on International Trade of Endangered Species (CITES)). No animals received placebo as an historical evaluation of colony health records showed that animals with confirmed ulcerative colitis which did not receive effective therapy continued to lose weight and showed further deterioration in their rectal biopsy pathology.

Methods

ANIMALS

All animals used in this study were captive bred at the University of Bristol colony. They were housed under standard environmental conditions as described previously.[24] [25] Animals were fed a complete pelleted diet (New World Primate diet, SDS Diets) and this was supplemented with fresh fruit each day. In addition, animals received regular supplementation with vitamin D3 administered orally.

Two animals were recruited to the pharmacokinetic study. Both appeared clinically normal and they were confirmed to be free of rectal pathology by histological examination of

a rectal biopsy specimen taken before recruitment. Six cottontop tamarins were recruited to the second phase of the study (Table I). All animals had confirmed ulcerative colitis, based on their clinical history of diarrhoea and weight loss, endoscopic examination of the colon along with histopathological examination of a rectal biopsy specimen taken at that time. Faecal samples were taken from the animals for culture to eliminate any known faecal pathogens. None of the six animals recruited to the study had received previous treatment for colitis.

MONOCLONAL ANTIBODY

Generation of the antibody

The antibody used in this study (CDP571) is derived from a murine monoclonal antibody to recombinant human TNFα which has been engineered to contain human g4 and κ light chain constant regions with Eu frameworks as described previously.[27]

Pharmacokinetics

Two animals received a single dose of CDP571 at 20 mg/kg by intramuscular injection. To administer the antibody, animals were sedated by an intramuscular injection of ketamine hydrochloride (Vetalar, Parke Davies; 20–25 mg/kg). Blood samples (maximum volume 0·5 ml into EDTA tubes) were taken from these animals before injection and at eight and 24 hours, and two, three, five, seven, 10, 14, 21, and 28 days after administration of CDP571. Samples were centrifuged and plasma stored at –70°C pending analysis for CDP571 and antibodies to CDP571.

Therapeutic protocol

Animals recruited to the study received CDP571 at a dose of 20 mg/kg every six days for a total of six doses. CDP571 was injected, by deep intramuscular injection, into the quadriceps femoris muscle under anaesthesia as described earlier (Table II).

EVALUATION OF RESPONSE TO TREATMENT

Standard indicators of disease progression in the cottontop tamarin were used to evaluate response to treatment. These were clinical signs, body weight and histopathological examination of rectal biopsy specimens taken on

TABLE I *Details of animals recruited to second part of the study*

Animal	Sex	Age at entering study
B221	Male	16 months
B213	Male	15 months
B201	Male	23 months
B111	Male	86 months
R194	Female	23 months
R192	Female	27 months

TABLE II *Outline of protocol*

Day	CDP571	Biopsy	Blood sample
–3		+	+
0	dose		
6	dose		
12	dose	+	+ (pre-drug)
18	dose		
24	dose		
25			+
30	dose		
32		+	+
49		+	+
63		+	+

TABLE III *Histology scoring system[26]*

Acute pathology	Score	Chronic pathology	Score
Polymorphonuclear cells in lamina propria	1	Mild chronic inflammation	1
Polymorphonuclear cells in crypt epithelium	2	Severe chronic inflammation	2
Crypt abscess	3		
Crypt destruction	4		
Overall maximum score	6		

days 12, 32, 49, and 63. Biopsy specimens were evaluated by a pathologist using an objective scoring system developed from one used previously for assessing human rectal specimens (Table III).[26] Specimens were graded from 0 (normal) to 6 (severe active disease) on the basis of inflammatory cell infiltrate, crypt architecture and mucosal disruption (Fig 1).

Biopsy scores and body weights before and after CDP571 treatment were compared using the Friedman non-parametric repeated measures test. Differences were considered significant when $p < 0.05$.

Blood samples were also taken when the animals were sedated for other procedures – that is, on days 12, 25, 32, 49, and 63, to permit measurement of plasma concentrations of CDP571 and of host antibodies to CDP571.

ELISAS FOR CDP571 AND ANTIBODIES TO CDP571

ELISA techniques were used as described previously.[27] Briefly, for the pharmacokinetic assay, diluted plasma samples were added to microtitre plates coated with rhTNFα and bound CDP571 was revealed with mouse anti-human IgG4 (Serotec) followed by horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jacksons Laboratories). Bound HRP was revealed using TMB substrate and colour development as proportional to the amount of CDP571 in the sample.

Antibodies to CDP571 were detected using a double antigen sandwich ELISA system. Plasma samples (diluted 1 in 10 in phosphate buffered saline)/1% bovine serum albumin (BSA)) were added to CDP571 coated plates and the sandwich was completed with CDP571 conjugated to HRP, followed by TMB substrate. An affinity purified rabbit hyperimmune serum was used as a standard and results expressed as units/ml (1 unit being equivalent to 1 mg/ml of the rabbit standard).

Results

PHARMACOKINETIC STUDY

Figure 2 shows the plasma elimination profiles for the two animals. Analyses demonstrated that CDP571 was cleared from normal tamarins with a half-life of around six days (5·8 and 7·0 days). By day 28, there was little host antibody response to CDP571. Therefore, the drug was administered every six days in the therapeutic protocol.

RESPONSE TO TREATMENT

None of the animals showed any adverse effects to repeated dosing with CDP571, either locally or systemically.

Analysis of plasma samples confirmed that during the treatment period, CDP571 concentrations continued to reach circulating levels in excess of 100 µg/ml (range 51·4–227·8 µg/ml; Table IV) and remained in circulation for several weeks (geometric mean of 0·5 µg/ml and range of 0·05–6·5 µg/ml at day 49). No immune response to the antibody was detected during the treatment period, although low levels of antibodies to CDP571 (3–9 units/ml) were seen in four of the six animals when CDP571 was cleared from the circulation after the final dose.

All animals showed a clinical response to treatment and in all cases there was an improvement in faecal quality after starting treatment. Figure 3 shows changes in body weight. Mean body weight rose following the first dose of antibody and remained significantly elevated during the treatment period and during the following month (+33·3 (10) g (mean (SEM)) at day 63, $p < 0.05$). This represents an increase of around 6% compared with pre-entry body weight and was maintained throughout the study.

Examination of rectal biopsy specimens showed a rapid fall in mean rectal biopsy score after starting treatment (Table V), indicating a
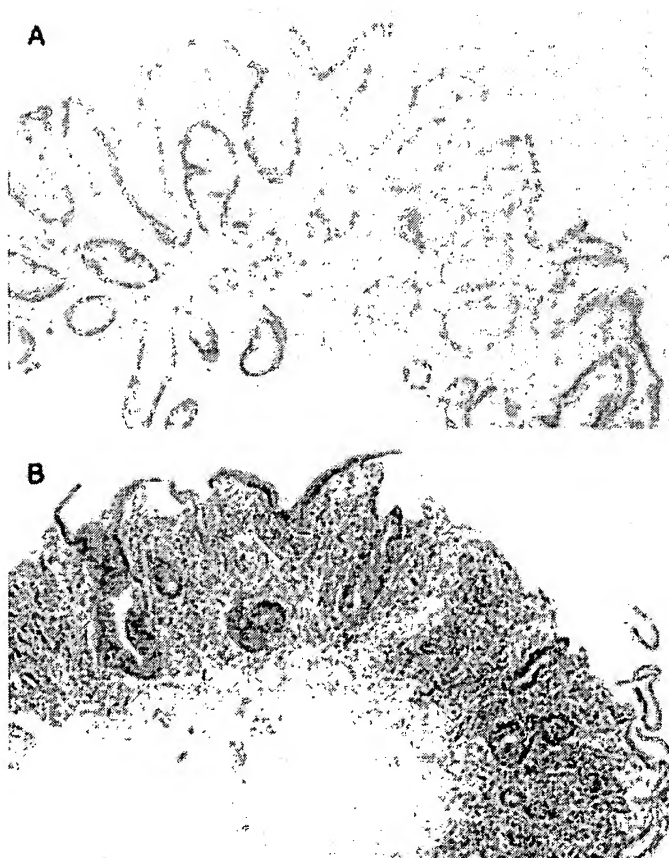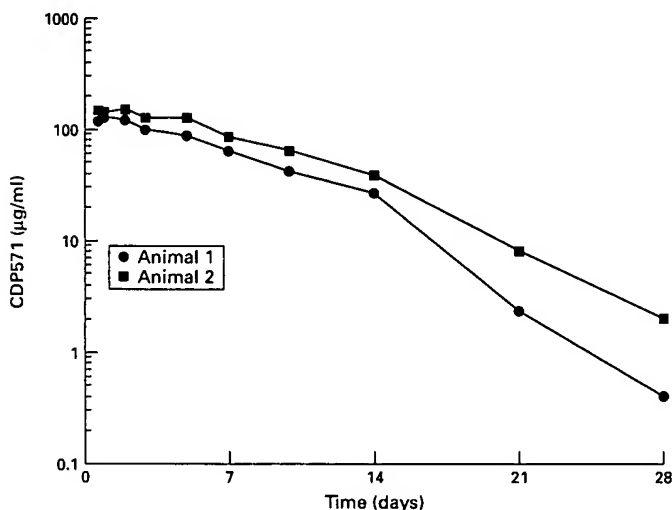

Figure 1: Representative histology of rectal biopsy specimens. (A) Grade 1 specimen with intact epithelium with few polymorphonuclear cells in the lamina propria. (B) Grade 5 specimen showing loss of epithelium, crypt destruction and infiltration of acute and chronic inflammatory cells.

Figure 2: *Plasma clearance profile of CDP571 in two cottontop tamarins after a single intramuscular injection of 20 mg/kg. Values are individual plasma concentrations.*

TABLE IV   *Plasma concentrations of CDP571 (μg/ml)*

Time after first dose (days)	Dose no./days after administration of CDP571	Animal number						Geometric mean
		R192	R194	B111	B201	B213	B221	
−3	Before	0·05*	0·05	0·05	0·05	0·05	NT	0·05
12	2/6	64·7	166·5	67·2	66·1	62·0	90·6	80·3
25	5/1	51·4	214·0	105·7	149·1	NT	227·8	131·6
32	6/2	6·3	172·5	57·9	68·0	37·2	154·5	53·9
49	6/19	0·05	4·2	0·84	0·31	0·05	6·5	0·5
63	6/33	0·05	0·05	0·05	0·05	0·05	1·21	0·1

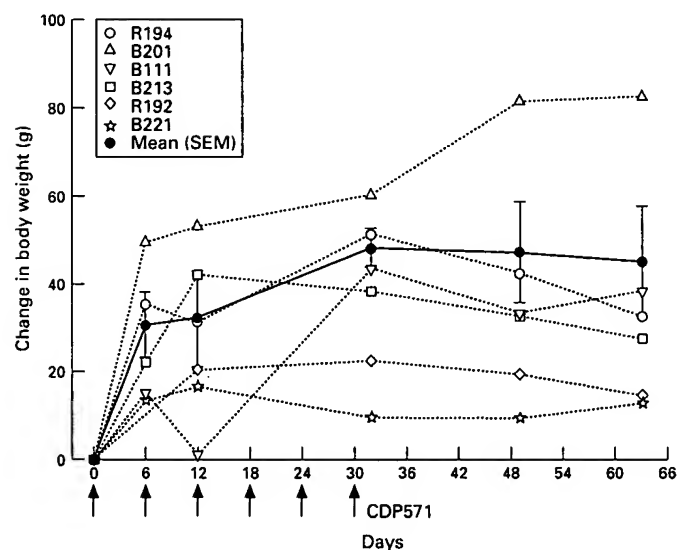*Limit of detection of 0·05 μg/ml.
NT=not tested.

Figure 3: *Changes in body weight for individual animals and the mean (SEM) (solid circles) value in cottontop tamarins given a course of CDP571 treatment (6# 20 mg/kg).*

TABLE V   *Colonic biopsy scores*

Time after first dose (days)	Biopsy score (mean (SEM))
−3	4·5 (0·2)
12	2·7 (0·8)*
32	3·0 (0·4)*
49	2·3 (0·7)*
63	3·8 (0·2)

Values are means (SEM) for six animals.
*$p < 0.05$ compared with pretreatment value (Friedman test).

value of 4·5 (0·2) to 3 (0·4) ($p < 0.05$). This improvement was maintained until day 49.

Discussion

Recent advances in the understanding of the mechanisms and control of inflammation have focused on the importance of numerous cytokines in this process. TNFα seems to play an important role in septic shock, rheumatoid arthritis and IBD. This has been demonstrated by the raised concentrations of TNFα both in plasma and locally in inflamed tissues in these conditions and has led to the development of monoclonal antibodies as potential therapies in these conditions. A murine antibody to TNFα has been used in early phase trials in patients with septic shock[28] but was associated with a significant host response to the antibody. However, antibody engineering involving either chimerisation or CDR grafting of murine monoclonal antibodies to TNFα to reduce immunogenicity has permitted certain conditions – for example, rheumatoid arthritis, to be treated.[12] [29] From this background, we evaluated the use of an antibody to TNFα in the treatment of ulcerative colitis in an animal model.

Previous work using chemically induced colonic inflammation in rats[17] has clearly demonstrated the ability of anti-murine TNFα to modulate the inflammatory response; however, the initial dose was given prior to the induction of inflammation. In ulcerative colitis in humans, treatment is required once disease is diagnosed and often the disease process may be longstanding before treatment is instituted.

Spontaneous ulcerative colitis in the cottontop tamarin provides a good model for evaluating new treatments for established disease in circumstances more related to the clinical situation. All animals recruited to this study had not received any previous treatment and the anti-TNFα was the sole treatment used. Based on the knowledge of pharmacokinetics of CDP571 and the lack of a deleterious immune response, we administered the antibody safely on a total of six occasions over six weeks. By comparison, Podolsky et al,[23] using a murine monoclonal antibody to α integrin to treat colitis in the cottontop tamarin, was restricted to therapy at two day intervals over 10 days because of the host immune response to the antibody. In this study, all animals demonstrated a rapid improvement after administration of anti-TNFα with an increase in body weight, an improvement in the consistency of stools and in rectal

reduction in disease activity. At day 12, there was already a statistically significant reduction in biopsy score compared with day 0, which by the end of treatment had fallen from a preentry

biopsy score. The response, which occurred as early as the first injection, was far more rapid than that seen to an established therapeutic regime based on the 5-aminosalycyclic acid compound olsalazine.[19] 5-Aminosalicylic acid based treatment is standard for ulcerative colitis in humans and has been used successfully to treat ulcerative colitis in the tamarin. However, when starting treatment there must be a gradual increase in dose levels. Improvement in rectal biopsy pathology and in clinical condition may not be seen for eight to 10 weeks in tamarins.

There were no placebo treated control animals in this study. The cottontop tamarin is listed under CITES as an endangered species and as such it was felt unethical to withhold therapy from animals with ulcerative colitis. Furthermore, it is known from previous studies in the colony that if animals are not treated the disease progresses unabated with progressive weight loss and deterioration of clinical condition leading either to death or euthanasia. Reference to colony records for six animals with confirmed ulcerative colitis that were not receiving therapy and were monitored over four weeks revealed an increase in mean rectal biopsy score from 1·3 to 2·6 over this period, indicating a deterioration in pathology.

Of the six animals that were recruited to the study, the majority (4/6) have not required any further treatment 18 months after the last dose. Two animals did, however, relapse and were given maintenance therapy with olsalazine. This occurred between eight months (R192) and one year (B213) after ceasing therapy. Prolonged benefit after a single course of therapy provides further encouragement for the use of anti-TNFα in the treatment of ulcerative colitis. The exact mechanism of action of anti-TNFα is uncertain in these animals, but it seems most likely that it inhibits the action of TNFα in the colon wall and so attenuates the inflammatory process.

Repeated therapy with CDP571 has been used in a small number of tamarins with ulcerative colitis which was refractory to standard therapy of olsalazine and prednisolone. Again, no adverse effects were recorded and although the animals showed some improvement in their condition, the clinical response was not as notable as that reported in this study of naive animals (unpublished observations). Human patients with rheumatoid arthritis have received repeated doses of chimeric anti-TNFα (cA2) and although apparently safe it was noted that the interval between doses fell as the number of doses administered rose.[30] By comparison, CDP571, at a dose of 10 mg/kg, has been given at two to three month intervals for a total of four doses in the treatment of rheumatoid arthritis. No adverse effects were noted and pharmacokinetic studies indicated no reduction in the half-life in patients.[31] These data provide encouragement for repeated dosing in humans.

The potential role of anti-TNFα in the treatment of human ulcerative colitis is now under investigation. Engineering of antibodies can prolong the half-life in humans from a few hours to one to two weeks.[27] Encouraging results have already been obtained with chimeric antibodies to TNFα in the treatment of Crohn's disease.[32] Studies with CDP571 are now continuing in acute and chronic inflammatory conditions such as IBD, and indeed preliminary studies with a single dose of antibody in an open study have proved encouraging.[33]

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